The Effect of Calcium Ion Concentration on The Lactic acid Production From Streptococcus Mutans Under Cariogenic Environment

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Abstract:

Objective: to investigate effect of calcium ions concentration on lactic acid production (mode of fermentation) from streptococcus mutans at excess glucose media (cariogenic environment). **Materials and methods:** Streptococcus mutans Clarke (ATCC 25175)was investigated in-vitro under glucose excess instances with different concentrations of glucose, at different calcium ion concentrations and also at different bacterial cell concentrations.The supernatants of the bacteria were analyzed using HPLC for lactic and acetic acids. **Results:** it was found that calcium ions can increase lactic acid or decrease it, and can encourage either mixed acid fermentation or homofermentation according to their proportion to glucose molecules and bacterial cell number.**Discussion:**to interpret these findings C-type lectins or C-type lectin-like molecules were suggested to be found on bacterial cell wall. These supposed molecules bind glucose molecules and calcium ions. Consequently when glucose molecules are introduced to the bacteria with high amounts they bind to these C-type lectins or the C-type lectin-like molecules using extracellular free calcium ions which become bonded to them. As a result of that,concentration of the free calcium ions decreases.This promotes lactic acid production as a major end product in an attempt to chelate calcium ions (from the supposed C-type lectins or the C-type lectin-like molecules or from tooth surface causing demineralization; caries) and restore the level of the free calcium ions extracellularly which restores mixed acid fermentation. **Conclusion:** mixed acid fermentation can be achieved at glucose excess medium (; Crabtree effect can be prevented) if calcium ion concentration was high enough in proportion to glucose molecules and number of bacterial cells. As well as pyruvate dehydrogenase enzyme can be activated in glucose excess medium when appropriate calcium ion concentration is provided. So, we mayuse S. mutans as a model to understand the mode of respiration in cancer cells (Warburg and Crabtree effects) which is very important in knowing carcinogenesis which may be due to the same mechanisms.

Introduction

Dental caries is a common dental disease, which affects the quality of life of people as well as causes many costs for treatment of it. It occurs, according to Miller's theory[1],due to lactic acid production and dissolution of the minerals of the salivary exposed dental hard tissues. Acid production occurs due to the fermentation of carbohydrates which come from diet or saliva. In a high sugar environment (as in carbohydrate intake), the mode of fermentation is directed almost toward lactic acid production by *lactate dehydrogenase enzyme(LDH)[2-4]* which is called *homofermentation*. But in a low sugar environment (as in resting dental plaque) the mode of fermentation is directed toward mixed acid fermentation viapyruvate dehydrogenaseenzyme(PDH) [5]

andpyruvate formate-lyase enzyme(PFL)[4, 6], which is extremely oxygen sensitive[7], and produce lactic acid with lower proportions, acetic acid, formic acid and ethanol which is called heterofermentation[4]. Lactate is more cariogenic alone than if it is produced in addition to acetic acidbecause in the latter case acetate will act as a buffer[8].

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Fermentation occurs intraorally essentially by bacteria[9-11]. Many types of bacteria found in dental plaque can produce lactic acid so the traditional non-specific plaque hypothesis[12] existed. But after the developments in technology, it was discovered that there are some types of bacteria more cariogenic than others so the specific plaque hypothesis[13] evolved. Then there was an update for the specific plaque hypothesis[14]. The cariogenicity of specific bacteria is determined by theiracidogenicity, acidouricity, and their ability to adhere to the tooth structure. Acidogenicity is the ability to produce acid whereas aciouricity is the ability to withstand low pH environments. The adherence to tooth structure occurs by extrapolysacchrides which produced by bacteria. And all these three criteria are found in streptococcus mutans. Whether it is true or not that only specific types of bacteria can produce dental caries,S. mutans and lactobacilli are certainly deeply involved in caries initiation and progression respectively[15].

The effect of the production of high amounts of lactic acid rather than aerobic oxidation in the presence of excess glucose is called the Crabtree effect[16]. This effect occurs in cancer cells which have intact mitochondria and capable of making aerobic oxidation of glucose molecule. And even in the presence of enough oxygen cancer cells prefer to make aerobic glycolysis and produce lactic acid which is known as the Warburg effect[17]. It is not known definitely why these cells behave like that although this

kind of respiration produces lesser amount of ATP molecules than complete oxidation of glucose molecule in aerobic respiration. This is considered one of the major signs of malignant cell transformation and upon this principle, Positron emission tomography works by detecting the site in the body of high glucose consumption as in cancer cells[18, 19].

Although S.mutans cannot do aerobic respiration because it lacks the genes required for that[20, 21] it uses the PDH enzyme to produce acetyl-CoA then transform it into ethanol, via alcohol dehydrogenase, or acetate, via acetate kinase.PDH enzyme is used in aerobic respiration in normal mammalian cells to produce acetyl-CoA which then enters the Kreps cycle for complete oxidation. The activity of this enzyme is inhibited in cancer cells[22]. So,there are similarities between S. mutans and cancer cells in the mode of metabolism of glucose in the presence of a low and high amount of it. So, we may use S. mutans as a model for the prediction of the cause of this behavior in cancer cells.

S. mutans occurs in dental plaque at tooth surfaces. Dental plaque has in its structure minerals like calcium, phosphorus, magnesium, strontium, fluoride, and lithium. The aqueous phase (plaque fluid) of dental plaque has more calcium ions than that found in saliva[23]. In many studies, it was found that there is a negative correlation between the amount of calcium in plaque and caries incidence[24-26]. It was found also that experimental dental caries can be prevented by mineralization of plaque[27]. The cause of this protective action is thought to be due to remineralization and the common ion effect. Also in an previous study of the effect ofEffect of consuming different dairy products on calcium, phosphorus and pH levels of human dental plaque it was found that there isastrong correlation between plaque pH and calcium levels[28]. During caries process, calcium ions increase inthe plaque fluid [25, 29] when lactic acid is produced and the calcium which is found in calcium sources in the solid phase of the plaque is reduced [29-32]. In a recent study, it was observed that extrapolysaccharides (which are considered one of the virulence factors of bacteria and the cause of the high cariogenic potential of sucrose) affect calcium flow and then affect the pathogenesis of caries[33]. So, free calcium ions are involved in the pathogenesis of dental caries. It is known also that calcium signaling is affected in cancer cells[34]. In this study, the importance of calcium ion for S.mutans bacteria was investigated to know the effect of calcium ion on the metabolism of S.mutans which then affect lactic acid production.

Materials and methods:

To know the effect of calcium ion concentration on the mode of fermentation and lactic acid production of S.mutans in the presence of a high sugar environment.

Materials:

Bacterial Strain:

Streptococcus mutans Clarke (ATCC 25175), serotype C was bought from microbial resources center (Cairo MIRCEN) and used in our study. The viability of the test strain was tested by monitoring their growth on Trypticase Soy Yeast Extract broth (TSYE) (Oxoid) under aerobic conditions.

Media:

-Trypticase Soy Yeast Extract broth (Oxoid): Composition: casein enzymichydrolysate, 17.0 g/L; dextrose, 2.5 g/L; dipotassium hydrogen phosphate, 2.5 g/L.

Preparation: 36 grams was suspended in 1000 ml heated distilled water till the medium dissolved completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

-Trypticase Soy Yeast Extract agar (Oxoid): Composition: casein enzymichydrolysate, 17.0 g/L; dextrose, 2.5 g/L; dipotassium hydrogen phosphate, 2.5 g/L; agar, 15.0 g/L.

Preparation: 51 grams was suspended in 1000 ml heated distilled water till the medium dissolved completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

-Brain Heart Infusion broth (BHI): Composition: Brain infusion solids, 12.5 g/L; Beef heart infusion solids, 5.0 g/L; Proteose peptone, 10.0 g/L; Glucose, 2.0 g/L; Sodium chloride, 5.0 g/L; Disodium phosphate, 2.5 g/L.

Preparation: 37 grams was suspended in 1000 ml heated distilled water till the medium dissolved completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Chemicals and Reagents:

1-Tetra butyl ammonium hydrogen sulphate obtained from Central Drug House, New Delhi, India, of 98% purity as stated by the manufacturer.
2-Acetonitrile Sigma-Aldrich, St.Lowis, Wasly USA.
3-Glacial acetic acid (96%) and Potassium chloride, ADWIC, Cairo, Egypt.
4-Lactic acid, El-Nasr Pharmaceutical Chemicals Co, Cairo, Egypt, 98%.
5-Potassium hexacyanoferrate (II), obtained from MERCK, Darmstadt, Germany, 99 %(Carrez I).
6-Zinc sulphatehepta hydrate, El-Nasr pharmaceutical chemicals Co, Cairo, Egypt (Carrez II).
7-Charcoal, activated, and produced by NICE Chemicals, kenela, India.
8-Calcium chloride dehydrate and Glucose anhydrous, extra pure, manufactured by ALPHA CHEMIKA, India.

Methods:

Microbiological method: The test strain was rehydrated in (TSYE) broth medium and incubated aerobically at 37 °C for 48 hours.

Bacterial growth obtained from broth culture was subcultured on (TSYE) agar plates and incubated aerobically for 48 hours at 37 °C.

Fresh colonies of S. mutans that obtained on solid medium were suspended in brain heart infusion broth in four concentrations (0.112, 0.267, 0.320, and 0.661) and optical densities of them were adjusted using a spectrophotometer at 600 nm wavelength.

Bacterial cell count of these concentrations was defined by viable colony count to make a relationship between cell count and optical densities of bacteria at these conditions.

For each concentration, 6 ml of bacterial cell suspension was added to 3 ml of manually prepared and filtered (using 0.22um MS ® CA syringe filter) 40% glucose solution and 3 ml of manually prepared (filtered by the same method) cacl2 of concentrations of (zero- 8- 16- 24- 32- 40) mmol and only one group KCL of 20mmol at 15 ml falcon tubes. The final concentrations of cacl2 were (zero, 2, 4, 6, 8, 10) mmol, 10% glucose, and 20 mmol KCL. The final optical densities of bacteria were (0.028, 0.071, 0.175, and 0.330).

All tubes were incubated aerobically at 37 c for 24 hours and the contents of the tubes were agitated and then the optical density of 1ml of all-tube was determined using a spectrophotometer at 600 nm.

Tubes were centrifuged at 1100 g for 10 minutes and the supernatants of bacteria were transferred by pipetting them to another sterile 15 ml falcon tubes then they were preserved at 4 c refrigerator until the next day for preparation and analysis using HPLC.

On another day the same bacteria were used in a concentration of optical density 0.124 at 600nm. Then the same previous procedures were done except that glucose concentration of one category of the two categories was 10 % and cacl2 concentrations were (80- 160- 320) mmol and KCL 640 mmol. The final concentrations will be 2.5% glucose, (20- 40- 80) mmol of CaCL2 and 160 mmol of Kcl. In the other category readymade glucose of 5% concentration was used as well as cacl2 concentrations were (16- 32- 40-80- 160- 320) mmol KCL 80 mmol to the final concentrations were (4- 8- 10- 20- 40- 80) mmol of cacl2, 20 mmol KCL and 1.25%glucose. The final optical density of bacteria was 0.055 determined by the same method.

HPLC Method:

HPLC instrument: HPLC measurements were performed with "Shimadzu LC-20 AD Prominence liquid chromatograph" equipped with a Rheodyne injector ralre with a 20 µL Loop and a SPD-20 A UV.
Mobile phase was filtered using Millipore filter Sibata and degassed using prominence degasser DGLL 20 Å 5.
A consort, P-901, Belguim pH – meter (Belgium) was used for pH measurements.

Chromatographic conditions:

A promosil RP C18 column (5um, 100 Å, 4.6x250mm) was used in this study. The mobile phase consisted of acetonitrile: 6 mM tetra butyl ammonium hydrogen sulphate 5:95 the final pH was 2.39.
The flow rate was 1mL/minute at ambient temperature. The detection was affected spectrophotometrically at 210nm.

Standard stock solutions:

Standard solutions of each of lactic acid and acetic acid were prepared as follows: 1% (v/v) (molarity of them are: 135990 nmol/mL and 167860 nmol/mL respectively) stock solutions of each were prepared by transferring accurately 1.0 mL of the original solution into 100 ml measuring flask then completed to the marke with distilled water and then filtered with Micropore filter paper of pore size 0.45 µm.

Working solutions:

Working solutions were prepared by transferring accurately suitable aliquots of the stock solution into 1.5 mL Eppendorff tubes and adding accurately measured volume of distelled water to get 1.0 mL.

preparation of samples:

9 mL of the sample was completed to 60 mL then 5 ml of Carrez I (85 Mm potassium hexacyanoferrate II) solution and 5 ml of Carrez II (250 mM zinc sulphate)solution were added and the pH was adjusted to 8 using 0.1 mMNaOH solution then completed with distilled water until the mark in 100 mL measuring flask then 1 gram of activated char coal was added to them and the mixture was agitated and then filtered with 0.45 µm filter as the method of extraction in the literature[35]. The blank (the BHI broth media without bacteria) was prepared with the same method.

Construction of calibration curves:

20µL aliquots of the filtrate were injected into the HPLC chromatogram under the following conditions: flow rate 1 mL/minute and the detector was set at 210 nm. Temperature was ambient. The peak area was plotted versus the final concentration of the acid (nmol) to get the calibration curves. Alternatively, the corresponding regression equations were derived.

Methods of statistics:

Data were statistically tested with the Statistical Package of Social Science (SPSS) program for Windows (Standard version 24).
Data were examined for normality with one-sample Kolmogorov-Smirnov test.
Continuous variables were demonstrated in the form of mean ± SD (standard deviation) for parametric data. Multiple groups were tested for significance difference with one way ANOVA test and in-between groups' comparisons were tested by post hoc LSD test.

Pearson correlation was used to correlate continuous data.

The significance level:

The level of significance (p-value) was at 5% for all the employed tests.
The difference was considered significant when the probability value was equal or less than 5% ($p \leq 0.05$).
The lower the p-value, the higher the significance of the difference
between compared groups.

Results:

10% glucose, $CaCL_2$ concentrations between 2 mmol and 10 mmol and initial optical densities are (0.1, 0.2, 0.3, and 0.6):

Optical density 0.1:
-Lactic acid and acetic acid increases significantly with increase in $CaCL_2$ concentrations except in concentrations 4mmol and 8 mmol in which acetic acid increases but not significantly more than the control.
The increase in the acetic acid is not with the same proportion of lactic acid so, the proportion between lactic acid and acetic acid increased significantly with increase in $CaCL_2$ concentrations except the 2 mmol $CaCL_2$ group in which it decrease but not significantly and decreased significantly in 6 mmol $CaCL_2$.
-In 20 mmol KCL group: lactic acid decreased significantly than the control and 10 mmol $CaCL_2$ groups in the same time acetic acid increased significantly than control and 10 mmol $CaCL_2$ groups so, the ratio between lactic and acetic acid decreased significantly than the control and 10 mmol $CaCL_2$ groups.
0.2 optical density:
-Lactic acid increases significantly with increase in $CaCL_2$ concentrations except in concentrations 2mmol and 6 mmol in which it increases but not significantly more than the control.
-In comparison to the control group acetic acid decreased significantly only in 2 mmol and 6 mmol groups while there was no significant change occurred in the rest of the groups of $CaCL_2$.
-The ratio between lactic acid and acetic acid increases significantly with increase in $CaCL_2$ concentrations.
-In 20 mmol KCL, lactic acid increased but not significantly more than the control group while that acetic acid decreased significantly more than the control significantly less than 10 mmol $CaCL_2$ group.
0.3 optical density:
-Lactic acid: no significant change except in 10 mmol $CaCL_2$ it increased significantly.
-Acetic acid: no significant change occurred except in 8 mmol $CaCL_2$ it increased significantly.
-Lactic acid/acetic acid ratio: increased significantly more than the control group except 8mmol $CaCL_2$ group it decreased significantly.
-In 20 mmol KCL group lactic acid, acetic acid and lactic acid/acetic acid ratio decreased significantly less than the control and 10 mmol $CaCL_2$ group.
0.6 optical density:
-Lactic acid: no significant change occurred except in 4 mmol $CaCL_2$ group it increased significantly.
-Acetic acid: no significant change occurred except 2 mmol $CaCL_2$ group it decreased significantly.
-Lactic acid/acetic acid ratio: increased significantly in some groups (2 mmol, 4 mmol $CaCL_2$) and decreased significantly in the others.
-In 20 mmol KCL lactic acid decreased significantly, acetic acid increased significantly and the lactic acid/acetic acid ratio decreased significantly in comparison to the control group and 10 mmol $CaCL_2$ group.
1.25% glucose and initial optical density is 0.124:
A.$CaCL_2$ concentration between(2mmol and 10 mmol):
-Lactic acid: increased significantly with the increase in $CaCL_2$ concentration.
-Acetic acid: increased significantly with the increase in $CaCL_2$ concentration.
-Lactic acid/acetic acid ratio: decreased significantly with increase in cacl2 concentration.
-In 20 mmol KCL group: lactic acid and acetic acid increased significantly more than the control but significantly less than the 10mmol cacl2 group. The ratio decreased significantly less than the control and 10 mmol cacl2 groups.
B.$CaCL_2$ concentration between 20 mmol and 80 mmol:
-Lactic acid: decreased significantly less than the control group.
-Acetic acid: increased significantly more than the control group and significantly less than 10 mmol cacl2 group.
-Lactic acid/acetic acid ratio: decreased significantly in comparison to the control and 10 mmol $CaCL_2$ groups.
2.25% glucose, $CaCL_2$ between 20mmol and 80mmol:
-Lactic acid: decreased significantly in comparison to the control group.
-Acetic acid: decreased significantly in comparison to the control group except in 40 mmol $CaCL_2$ group.
-Lactic acid/acetic acid ratio: decreased significantly in comparison to the control group.
-In 160 mmol KCL group: lactic acid decreased significantly in comparison to the control group but it was significantly more than 80 mmol $CaCL_2$ group. Acetic acid was significantly higher than the control and 80 mmol groups. Lactic acid/acetic acid ratio was significantly lower than the control but significantly higher than 80 mmol $CaCL_2$.

System suitability tests:

Compound	NTP	HETP	Ḱ	α	Rs
Lactic acid	4373	0.0058	2.415	1.128	1.205
Acetic acid	5855	0.0043	2.726		

NTP: number of theoretical plates.
HETP: height equivalent to theoretical plates.
Ḱ: capacity factor.
α: selectivity factor.
RS: resolution factor.

Discussion:

It appears clearly from all these experiments that when glucose was very high as in the case of 10% glucose and calcium ions concentration was between 2 mmol and 10mmol (normal calcium ions in dental plaque is between1 and 10 mM in resting dental plaque[29] and after a pH drop[25]) the metabolism of theS. mutans bacteria inThis different concentration was directed towards homolactic fermentation and lactic acid increased with the increase in calcium ion in the same time the ratio between lactic and acetic acid increased. This indirectly indicates that PDH enzyme activity was inhibited in the same time LDH enzyme was activated.

It is worth noting that these all experiments were done under aerobic conditions so, the acetate produced was due to PDH enzyme not PFL enzyme which is extremely sensitive to oxygen.

But when glucose was reduced to 2.25% and the lowest concentration of bacteria used in 10% glucose experiment have been used in the same time calcium ions increased more than found normally in dental plaque (concentrations were between 20 mmol and 80 mmol) the metabolism is generally reduced and directed towards mixed acid fermentation because the decrease in acetic acid is not with the same proportion of the decrease of lactic acid. So, PDH enzyme activity was increased.

When glucose was reduced to 1.25% and the lowest bacterial concentration used in 10% glucose experiments was used metabolism increased and directed towards mixed acid fermentation when the concentration of calcium was between 2 mmol and 10 mmol but when calcium ions increased more than that (20 mmol to 80mmol) the metabolism is reduced and also directed towards mixed acid fermentation.

From these experiments we can see that mixed acid Bec fermentation (and accordingly increase in PDH enzyme activity) can occurs even in high sugar environments (as in 2.25%, and 1.25% glucose) if there are enough calcium ions in relation to glucose molecules. And can be inhibited if the glucose molecules is higher in relation to the same concentrations of calcium ions.

To interpret the link between calcium ions and glucose molecules, we can imagine that there are molecules on the surface of bacterial cell wall these molecules can bind glucose molecules and need calcium ions to do that. This description is identical to the C-type lectins. C-type lectins are proteins which can bind carbohydrates and need calcium ions to do that[36]. So, upon that When bacterial cells are found with certain concentration and glucose molecules are introduced glucose molecules bind to C-type lectin molecules which are found on bacterial cell wall surface and also calcium ions bind to them which lowers free extracellar calcium ions to the extent which induce homofermentation so lactic acid is produced as the major product of fermentation and PDH enzyme is inhibited. But if calcium ions were introduced with glucose molecules the metabolism can be accelerated and directed towards homofermentation when glucose molecules is very high in relation to calcium ion molecules (as in 10% glucose) or can be inhibited and directed towards mixed acid fermentation or accelerated and directed also towards mixed acid fermentation it was described before.

explains the carbetree effect in s.mutans that in resting dental plaque there is low sugar environment so, free calcium ions are found with enough quantities therefore acetic acid is dominated*due to mixed acid fermentation and no caries occurs at these conditions. But when sugar is introduced with high amounts to dental plaque bacteria glucose molecules will bind with high concentration to the supposed C-type lectins and bind with them normally found calcium ions in the plaque fluid which decrease greatly extracellular calcium ions and induce bacterial cells to produce lactic acid as the major product of fermentation (homofermentation) in an attempt to chelate calcium ions and restore the suitable calcium level in dental plaque fluid. This causes dissolution of calcium ions found in dental hard tissues; caries. These calcium ions replenish calcium sources in dental plaque, restore the normal level of calcium ions in plaque fluid and the excess of calcium ions is lost to thesaliva acchording the concentration gradient. So, accordingly saliva is essential for caries process to occur! Because if there is complete absence of saliva the calcium ions will not be lost and they will be concentrated in dental plaque and the mode of fermentation will be mixed acid fermentation and caries will not occur. It is clear that when there is xerostomia rampant caries occurs. But if we look at the clinical picture of caries in xerostomia we will find that carious lesions are mainly in the cervix of teeth where the saliva retained. This little remaining saliva may come from the minor salivary glands or from the remaining functioning part of the diseased salivary gland.

because the number of C-type lectin is very important in the determination of the type of the reaction which will occur and these C-type lectins are found on the bacterial cell wall surfaces number of bacteria is very important for caries to occur and this agree with the study of[37].

In vivo bacterial cells are attached to each other and also to the extracellular matrix in dental plaque which lowers the available sites of C-type lectin proteins .

In the present study planktonic bacterial cells were used so, the comparison between the number of bacteria in dental plaque and that have been used in this study will not be effective.

So, caries protective action of calcium is because of the maintenance of mixed acid fermentation either with increase in lactic acid but in the same time increase of acetic acid which acts as buffer or by decrease both lactic and acetic acid and maintain mixed acid fermentation. This

depends on the number of available sites of the C- type lectins in dental plaque bacteria.
Fluoride action may also be due to the ability of fluoride ion to bind calcium ions as is now thought that calcium fluoride complex is responsible for the caries protective action of fluoride[38]. Because the fluoride concentration found in dental plaque is not enough for prevention of acid production from bacteria[39].

It was previously found also that xylitol which is sugar alcohol can change the mode of fermentation of S. mutans bacteria[40]. Xylitol is capable of binding calcium ions[41] which also may the cause for its effect. It is worth noting that xylitol has anticancer effect as found in a recent study[42].

We may also apply this concept on cancer cell mode of respiration. Cancer cells alter their mode of respiration to lactic acid production in the presence of oxygen this may be as a result of the decrease in the extracellular calcium ions which may be due to alterations in the number of cell surface proteins (may be C-type lectins) which bind glucose and calcium ions or due to any cause that locally decreases the free extracellar calcium ions. This prospective agree with that was found in previous study that lactate acidosis caused by cancer cells restore the aerobic respiration in these cells[43]. This may be due to the ability of lactic acid to chelate calcium ions and increase their concentration in the extracellular fluid which restore the function of PDH enzyme and the normal aerobic respiration.
It was also found that stress caused bylactic acid inS. mutansincreases the activity of alpha PDH enzyme[44].

Conclusion:

Extracellular calcium ion concentration affect the mode and the amount of fermentation according to its proportion to glucose molecule and bacterial cell number. This explains Crabtree effect and how caries process occurs.

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