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The Development of Nitrate Reductase in Chlorella and its Repression by Ammonium

By
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With 7 Figures in the Text

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Chlorella vulgaris growing with ammonium nitrate as nitrogen source utilises all of the ammonium-N before nitrate begins to be assimilated. We have attempted to analyse this phenomenon and have shown that the assimilation of ammonium in some way inhibits nitrate reduction (Syrett and Morris 1963). In this paper a second effect of ammonium is established and discussed, namely the repression, by ammonium, of the development of nitrate reductase activity.

Methods

Chlorella vulgaris (Pearsall's strain) was grown as described in the preceding paper (Syrett and Morris 1963). Unless otherwise stated all experimental methods were as described in that paper.

Measurement of nitrate reductase activity

Cell-free extracts were prepared as in the previous paper. When it was necessary to store cells before preparing extracts, the cells were washed and suspended in 0.1 M Tris buffer, p_H 7.4 and stored at $-14°$ for 24—28 h. After cell-free extracts had been prepared, they were assayed for nitrate reductase activity at once; the extracts contained no detectable nitrite reductase activity.

The reaction mixture for nitrate reductase assay contained, in 1.5 ml, 20 μ moles potassium nitrate, 20 μ moles Tris, p_H 7.4, 0.2 mgs DPNH (reduced sodium salt) and cell free extract (in 0.1 M Tris buffer) containing 0.5—1.0 mg of protein. DPNH was added at zero time and the mixture was incubated at 28—29° for 10 min. Blanks without DPNH were always included. At the end of the reaction time, residual DPNH was removed by ethanol-barium acetate treatment (Medina and Nicholas 1957). The tubes containing the reaction mixture were immersed in iced water, 2.5 mls of ice-cold 95% ethanol were added followed by 0.1 ml of M barium acetate. The tubes were agitated, centrifuged and nitrite in the supernatant estimated with Griess-Ilosvay reagent (Syrett and Morris 1963). Low and erroneous values were obtained if the residual DPNH was not removed. Nitrite standards were prepared with the same barium acetate-ethanol treatment. Under these assay conditions nitrate reduction proceeded linearly with time and the rate of reduction was proportional to the quantity of protein added. Nitrite formed after 10 min was taken as a measure of nitrate reductase activity. In this paper, nitrate reductase activity is expressed as mμ moles nitrite formed per mg protein

per minute. Protein was measured by the Lowry method (LOWRY et al. 1951). It was found that 0.1 M Tris, in which the enzyme was extracted, interferes with the Lowry reaction. Bovine albumin standards were therefore prepared with 0.1 M Tris present.

Results

1. The effect of nitrogen source on nitrate reductase activity

Cell-free extracts from cells of *Chlorella vulgaris* grown with potassium nitrate as nitrogen source possess much nitrate reductase activity, whereas extracts of cells grown with ammonium sulphate possess little. There is an intermediary level of nitrate reductase in extracts of cells grown with ammonium nitrate as nitrogen source (Table 1).

Table 1. *Nitrate reductase activity in cell-free extracts of cells grown with different nitrogen sources*
The cells grown with ammonium nitrate were harvested before all the ammonium had disappeared from the culture medium. The results of two separate experiments are shown. Each figure is the mean of two independent assays. Note that the activity in ammonium nitrate cells varies and is about 38—65% of that in potassium nitrate cells

Nitrogen source for growth	Specific activity of nitrate reductase (mμ moles nitrite per mg protein per min)	
	Expt. 1	Expt. 2
KNO_3	32.5	36.9
NH_4NO_3	21.0	13.9
$(NH_4)_2SO_4$	0.7	1.0

A comparison of the nitrate reductase activity of nitrate-grown cells with their maximum growth rate is of interest. Generally, the reductase activity in these extracts is approx. 30 mμ moles nitrite formed per mg protein per minute, or 1.8 μ moles nitrite per mg protein per hour i.e. 1 mg protein reduces about 25 μg of nitrate-N in one hour. Since 1 mg of protein contains about 160 μg N, the activity of the reductase is such that it could reduce enough nitrate-N to double the protein content of the cell-free extract in 6—7 h. This time compares favourably with the minimum generation time of *C. vulgaris* which is about 8 h. It appears, therefore, that, in these experiments, the activity of the reductase in the cell-free extract, is sufficient to account for the rate of growth of *C. vulgaris* with nitrate as nitrogen-source. This agreement may be fortuitous, however, since in a subsequent paper we shall show that nitrogen-starved cells contain considerably less nitrate reductase activity and yet assimilate nitrate rapidly.

In the preceding paper (SYRETT and MORRIS 1963), we suggested that cells grown on ammonium nitrate contain an inhibitor of nitrate reductase activity. Such an inhibitor in cell-free extracts would, of course, affect results like those in Table 1. The nitrate reductase activity of extracts from cells grown on potassium nitrate or ammonium nitrate was therefore assayed after mixing the extracts with those from ammonium

nitrate or ammonium sulphate grown cells. The results showed that, when mixed, the activities of the extracts were additive within the limits of experimental error. Therefore no inhibitor can be present, in effective concentration, in the extracts from ammonium nitrate and ammonium sulphate grown cells and consequently the results in Table 1 represent real differences of nitrate reductase activity in the extracts.

2. Increase of nitrate reductase activity during incubation with nitrate

Cells grown with potassium nitrate as nitrogen source assimilate nitrate immediately it is supplied to them. In contrast, cells grown with ammonium sulphate as nitrogen source do not assimilate nitrate until after a lag period of about two hours (Fig. 1). Nitrate reductase activity in ammonium sulphate grown cells is initially low but increases markedly during incubation with nitrate (Fig. 2) and after two hours the activity has increased by 10—20 times. The two hour lag in nitrate assimilation must be due, in part, to the low initial level of nitrate reductase but there is also evidence that ammonium-grown cells contain an inhibitor of nitrate assimilation which is only slowly removed (Syrett and Morris 1963).

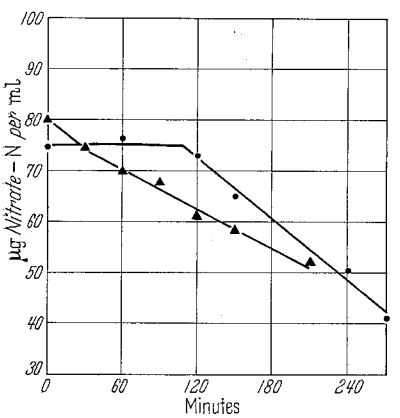

Fig. 1

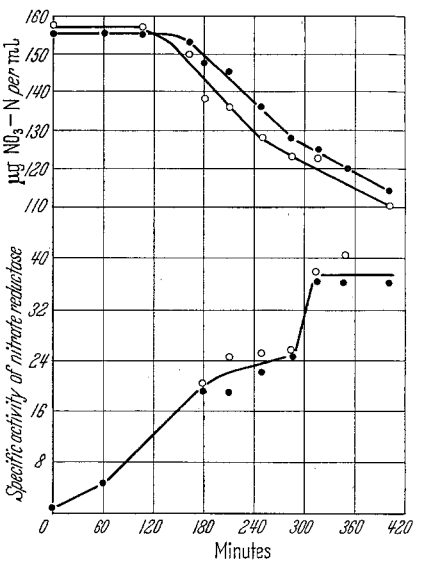

Fig. 2

Fig. 1. The assimilation of nitrate by ammonium-grown cells (●——●) and nitrate-grown cells (▲——▲). Incubation in darkness with 1% (w/v) glucose present. Temperature, 25°; pH 6.1; cell density, 5.0 mg dry wt./ml

Fig. 2. The development of nitrate reductase activity (lower) and the assimilation of nitrate (upper) when ammonium-grown cells are transferred to medium containing potassium nitrate. The results of duplicate cultures are shown. Incubation in darkness with 1% (w/v) glucose present. Temperature, 25°; pH 6.1; cell density, 4.8 mg dry wt. per ml. Counts of cell numbers showed that there was no significant increase during the course of the experiment

In earlier experiments the increase in nitrate reductase activity appeared to follow a sigmoid curve and reach a plateau value after three hours. The enzyme level so attained was always lower than that found in cells grown for several generations with potassium nitrate as nitrogen source. Fig. 2 shows the reason for this difference. The rate of increase of nitrate reductase activity does, indeed, slow down after three to four hours but subsequently, at about the fifth hour, a further and rapid increase in activity occurs and the enzyme level attains that of potassium nitrate grown cells (cf. Table 1 and Fig. 2). This rather complex and unexpected time course has been well established by several experiments but the reasons for it are not understood. It may have a connection with the time course of nitrate assimilation because the rate of assimilation appears to slow down after 4—5 hours and, at this time, the second increase in nitrate reductase activity takes place.

3. The effect of chloramphenicol and p-fluorophenylalanine on the increase in nitrate reductase activity

The increase of nitrate reductase activity following incubation of ammonium-grown cells with nitrate is partially prevented by either chloramphenicol or p-fluorophenylalanine (Fig. 3). The effective concentration of chloramphenicol, while considerably higher than that

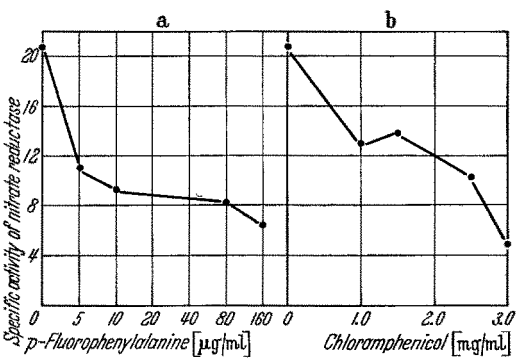

Fig. 3a and b. The effect of *p*-fluorophenylalanine (a) and chloramphenicol (b) on the development of nitrate reductase activity when ammonium-grown cells are transferred to medium containing nitrate. Incubation at 25° in darkness with 1% (w/v) glucose present; pH 6.1. The initial concentration of nitrate was 5 mM. Cell density 6.0 mg dry wt. per ml. Nitrate reductase activity was assayed after three hrs. incubation of the cells with nitrate and inhibitor

required to prevent protein synthesis in bacteria, is similar to that inhibiting amino-acid incorporation into microsomal preparations from higher plants (Rabson and Novelli 1960). We have shown, too, that the concentrations of chloramphenicol and p-fluorophenylalanine which prevent the development of nitrate reductase activity, lower the incorporation of ^{14}C from [^{14}C] glucose or [^{14}C] phenylalanine into the

protein fraction of *Chlorella*. Thus one can conclude that chloramphenicol and p-fluorophenylalanine are inhibiting the synthesis of active protein and that at least part of the increase in nitrate reductase activity involves the *de novo* synthesis of protein.

In an experiment in which nitrate reductase activity was allowed to develop as shown in Fig. 2, the presence of chloramphenicol (3 mg/ml) inhibited the development of reductase activity by 40% after three hours and by 45% after six hours, thus both phases of the induction curve were equally affected by chloramphenicol.

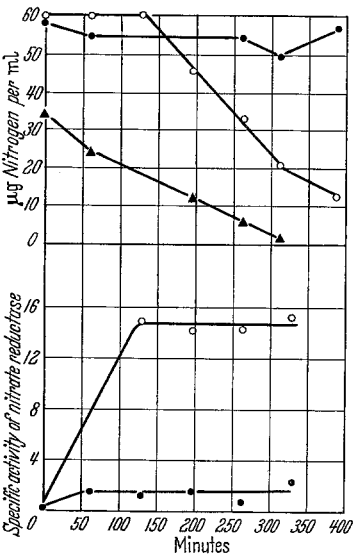

Fig. 4

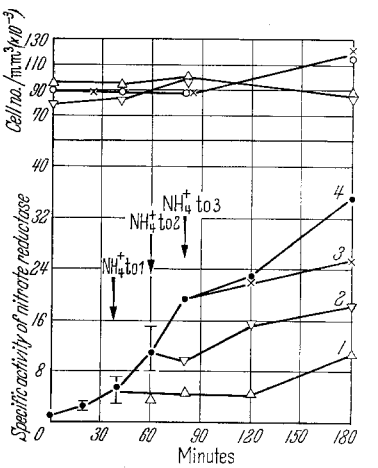

Fig. 5

Fig. 4. The effect of ammonium on the development of nitrate reductase activity. Lower; the development of nitrate reductase activity in presence (●———●) and absence (○———○) of 2.5 mM ammonium. Upper; the assimilation of nitrate in presence (●———●) and absence (○———○) of 2.5 mM ammonium and the assimilation of ammonium (▲———▲). Cell density, 5.0 mg dry wt./ml. Incubation in darkness at 25° with 1% (w/v) glucose. pH 6.1. The cell number did not change significantly during the experiment

Fig. 5. The effect of ammonium on the development of nitrate reductase activity. Ammonium-grown cells were transferred to a medium containing 5 mM potassium nitrate and 1%/w/v glucose; pH 6.1. They were incubated in darkness at 25°. Cultures *1*, *2* and *3* received ammonium (final concentration, 5 mM) at the times indicated. The lower figure shows the time course of the development of nitrate reductase activity in the cultures. The first three values are the mean values from three or four separate extractions of the enzyme; the extreme values are shown also. The upper figure shows that no significant change in cell number occurred during the experiment

4. The effect of ammonium on the development of nitrate reductase activity

The inclusion of a small quantity of ammonium in the medium prevents the development of nitrate reductase activity (Fig. 4); nitrate assimilation is also prevented.

When ammonium is added after the development of nitrate reductase activity has begun, further increase of activity is prevented (Fig. 5); ammonium is particularly effective when added near the start of the incubation period.

In the experiment shown in Fig. 4, the small quantity of ammonium added almost completely prevented the development of nitrate reductase activity. Nevertheless, cells grown for several generations with ammonium nitrate as nitrogen source do contain appreciable nitrate reductase activity (Table 1). The explanation is that the nitrate reductase activity of cells in the presence of ammonium can be increased by increasing the nitrate concentration (Fig. 6). In the growth medium containing ammonium nitrate the concentration of nitrate is 280 μg nitrate-N per ml.

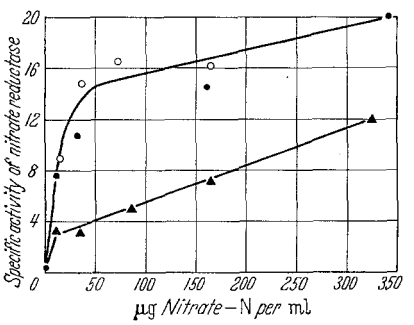

Fig. 6

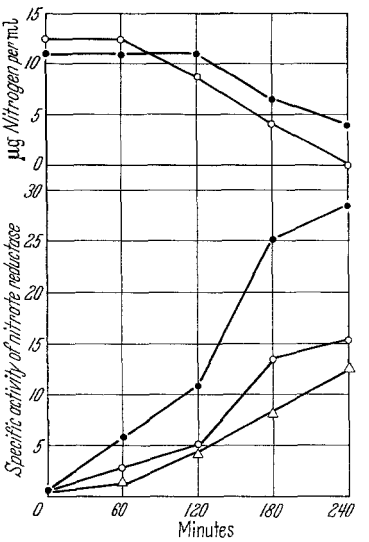

Fig. 7

Fig. 6. The effect of nitrate concentration on the development of nitrate reductase activity. Ammonium-grown cells were transferred to medium containing increasing concentrations of nitrate in the absence (circles) and presence (triangles) of ammonium. The results of two experiments in the absence of ammonium are shown. The initial ammonium concentration was 10 μg ammonium-N/ml and the final concentration about 3 μg ammonium-N per ml. Cell density 1.5 mg per ml. Temperature 25°. p_H 6.1. The cells were incubated for 3 hrs. in darkness in medium containing 1% (w/v) glucose; nitrate reductase activities were then measured

Fig. 7. Lower graph: the development of nitrate reductase activity in ammonium-grown cells transferred to nitrate (•———•), nitrite (○———○) or N-free (△———△) medium. Upper graph: the disappearance of nitrate (•———•) or nitrite (○———○). Cell density 2.0 mg dry wt. per ml. Incubation in darkness with 1% (w/v) glucose. p_H 6.1. Temperature 25°

5. *The development of nitrate reductase activity in the absence of nitrate*

The experiments of Figs. 2, 4 and 5 suggest that nitrate reductase in *Chlorella* is an enzyme whose formation is induced by nitrate and repressed by ammonium. However, considerable development of nitrate reductase activity is possible in the absence of nitrate providing that ammonium too, is absent. Fig. 7 shows that nitrate reductase activity increases when ammonium-grown cells are incubated in a nitrogen-free

medium. The increase in activity is considerable but not as great as that in cells incubated with nitrate. Incubation with nitrite gives an increase somewhat greater than that obtained in a nitrogen-free medium.

Such experiments suggest that cells grown with nitrogen sources not readily converted to ammonium might contain nitrate reductase activity even though the growth medium contained no nitrate. Suitable nitrogen sources are the simpler amino-acids and urea. Table 2 shows that cells grown with glycine or urea as nitrogen source can, in fact, contain about 20—40% of the nitrate reductase activity found in cells grown with potassium nitrate as nitrogen source although the levels tend to be variable. On the other hand, cells grown with alanine and arginine as nitrogen source contain little nitrate reductase. Ammonium accumulation could never be detected in any of these cultures. Nevertheless alanine and arginine may be more readily converted to ammonium than glycine and urea and so nitrate reductase formation repressed.

6. The effect of glucose on nitrate reductase formation

The results of Table 2 were obtained from cultures grown in the light and aerated with air enriched with carbon dioxide. In contrast, cells grown in darkness with glucose as carbon source and urea or glycine

Table 2. *Nitrate reductase activity in cellfree extracts from cells grown with various nitrogen sources*

All media contained 280 μg nitrogen per ml. Mean values for specific activity of nitrate reductase together with their standard errors and the number of experiments are given. Cells were harvested after 50 hrs. growth i.e. ca. 5—6 cell divisions had occurred. The little ammonium introduced into the growth medium with the inoculum was sufficient to allow less than a single division. The cultures were aerated with 0.5% carbon dioxide in air and illuminated with a tungsten filament lamp giving a light intensity of 600 foot candles. Temperature 25°, p_H 6.1

N-source	Specific activity of nitrate reductase	
$(NH_4)_2SO_4$	0	(6)
KNO_3	30.7 ± 2.12	(10)
L-Glycine	12.7 ± 2.46	(9)
DL-Alanine	2.0 ± 0.45	(5)
Urea	6.7 ± 1.75	(5)
L-Arginine-HCl	2.2 ± 0.57	(6)

as nitrogen source, contain no detectable nitrate reductase (Table 3). Moreover, when glucose is added, illuminated cultures fail to form nitrate reductase with glycine or urea as nitrogen source. The addition of glucose has little effect on the nitrate reductase of cells growing with potassium nitrate as nitrogen source.

Table 3. *The activity of nitrate reductase in cell-free extracts of cells grown with three nitrogen sources in the presence and absence of light and glucose*

Mean values and the number of experiments are given. Temperature, 25°. pH, 6.1. Condition 1. Light intensity, 600 foot candles; aeration with 0.5% carbon dioxide in air. Growth continued for about 50 hrs. i.e. about 5—6 cell divisions. Condition 2. Light intensity, 800 foot candles; growth for 88 hrs. i.e. about 5—6 cell divisions 1% (w/v) glucose present. Condition 3. Incubation in darkness, 1% (w/v) glucose present; growth for about 144 hrs. i.e. about 5—6 cell divisions. All media contained 280 μg nitrogen per ml

N-source	Specific activity of nitrate reductase (mμ moles nitrite per mg protein per min)		
	1 Light — glucose	2 Light + glucose	3 Dark + glucose
KNO_3	30.7 (10)	25.2 (1)	25.2 (4)
L-Glycine	12.7 (9)	0.7 (2)	0 (2)
Urea	6.7 (5)	0.9 (2)	0 (2)

Discussion

Chlorella cells grown with nitrate as nitrogen source contain much nitrate reductase activity whereas cells grown with ammonium-N contain very little (Table 1). There are reports of similar behaviour in bacteria (Pollock and Wainwright 1948), fungi (Nicholas, Nason and McElroy 1954; Morton 1956), algae (Kessler and Czygan 1963) and angiosperms (Hewitt and Afridi 1959).

When ammonium-grown *Chlorella* cells are incubated, in darkness, in a medium containing nitrate and glucose, nitrate reductase activity develops rapidly (Fig. 2). It can increase approximately 20 times in two hours and, by six hours, the activity may have reached 50—60 times the initial level. This increase in activity is partially prevented by chloramphenicol and by p-fluorophenylalanine. Since both these compounds are known to prevent the formation of active enzyme protein (Wisseman et al. 1954; Cohen and Munier 1959) it is probable that, at least, part of the increase in reductase activity involves the synthesis of protein.

The presence of nitrate and ammonium can affect the intracellular level of nitrate reductase in *Chlorella* in two distinct ways. Firstly, the formation of the enzyme is inhibited or repressed by the presence of small quantities of ammonium. The rapid increase in nitrate reductase activity following the incubation of ammonium grown cells with nitrate, ceases as soon as a small quantity of ammonium is added (Fig. 5) and, when a little ammonium is included in the medium initially, very little increase occurs (Fig. 4). In a previous paper (Syrett and Morris 1963) we showed that the nitrate assimilation of cells having a full complement of nitrate reductase, is inhibited when ammonium is added but

that ammonium must be assimilated for this inhibition to take place. It may also be true that ammonium must be assimilated before it represses the formation of nitrate reductase activity and that a compound formed from ammonium, rather than ammonium itself, is the repressor; we have no evidence on this point.

The second factor affecting the nitrate reductase activity of cells is the presence of nitrate in the medium. Nitrate is not essential for the development of nitrate reductase activity since cells grown in the complete absence of nitrate, with either glycine or urea as nitrogen sources, can contain appreciable activity (Table 2). Similarly nitrate reductase activity increases markedly when ammonium-grown cells are incubated in a nitrogen-free medium (Fig. 7). Nevertheless, when nitrate is included in the incubation medium, the increase in nitrate reductase activity is very much more rapid (Fig. 2 and 7) and, after two hours incubation, the enzyme level is more than twice that in nitrogen-starved cells. Nitrate could increase enzyme formation in one of two ways. Firstly, it might stimulate protein synthesis by serving as a nitrogen source from which free ammonium is not readily formed, or, secondly, it might act as an inducer of nitrate reductase formation. The evidence indicates that it acts as an inducer since during the initial two hour period when nitrate reductase activity increases so greatly, nitrate is not assimilated from the medium (Fig. 1 and 2) and hence is not available as a nitrogen source for protein synthesis. That nitrate has an inducing effect is also shown by the interaction between ammonium and nitrate concentration (Fig. 6). When the nitrate concentration is low, the addition of $5 \cdot 10^{-4}$ M ammonium greatly depresses the formation of nitrate reductase activity but, on raising the nitrate concentration to $2 \cdot 10^{-2}$ M, i.e. 40 times the concentration of ammonium, an appreciable quantity of nitrate reductase is formed. This result probably explains why *Chlorella* and other organisms, grown with ammonium nitrate as nitrogen source, usually contain appreciable nitrate reductase activity (Table 1). In such cultures, the concentrations of ammonium-N and nitrate-N are equal initially but since ammonium is assimilated preferentially (SYRETT and MORRIS 1963), the ratio of nitrate to ammonium increases as the culture grows.

Thus we can conclude that, while nitrate reductase can be formed in the absence of nitrate, its formation is stimulated or induced by nitrate and inhibited or repressed by ammonium or some compound derived from it. The interaction of these two effects may be the cause of the rather complex kinetics of enzyme formation when ammonium-grown cells are incubated with nitrate and glucose in darkness (Fig. 2), for in such an experiment, both induction by nitrate and removal of ammonium repression will operate.

Summary

Chlorella vulgaris, grown with ammonium sulphate as nitrogen source, contains very little nitrate reductase activity in contrast to cells grown with potassium nitrate. When ammonium-grown cells are transferred to a nitrate medium, nitrate reductase activity increases rapidly and the increase is partially prevented by chloramphenicol and by p-fluorophenylalanine, suggesting that protein synthesis is involved. The increase in nitrate reductase activity is prevented by small quantities of ammonium; this inhibition is overcome, in part, by raising the concentration of nitrate. Although nitrate stimulates the development of nitrate reductase activity, its presence is not essential for the formation of the enzyme since this is formed when ammonium-grown cells are starved of nitrogen and when cells are grown with urea or glycine as nitrogen source. It is concluded that the formation of the enzyme is stimulated (induced) by nitrate and inhibited (repressed) by ammonium.

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