The effect of Norflurazon on photosynthetic activity in isolated thylakoids from wheat (*Triticum aestivum* L.)

Aferdita Hoti
Table of contents

Summary ......................................................................................................................... 1

Abbreviations .................................................................................................................. 1

1 Introduction ................................................................................................................. 2

2 Materials and methods ............................................................................................... 5

2.1 Growth conditions .................................................................................................... 5

2.2 Chloroplast and thylakoid isolation ......................................................................... 5

2.3 PSI-activity .............................................................................................................. 6

2.4 PSII-activity ............................................................................................................. 6

2.5 SDS-PAGE .............................................................................................................. 6

3 Results ....................................................................................................................... 7

3.1 Chlorophyll/plastid ................................................................................................. 7

3.2 ................................................................................................................................. 8

3.3 PSI activity .............................................................................................................. 8

3.4 ................................................................................................................................. 9

3.5 SDS-PAGE ............................................................................................................. 9

3.6 ................................................................................................................................. 10

4 Discussion .................................................................................................................. 10

References ..................................................................................................................... 12

5 Appendix ..................................................................................................................... 14
Summary

This investigation has been performed on Norflurazon (NF) treated young wheat plants with emphasis on their capacity to perform light-driven photosynthesis, as an effect of carotenoid deficiency. The wheat seeds were soaked for approximately 17 hour in tap water or in water containing NF (28mg l\(^{-1}\) ca 10\(^{-4}\) M). Seedlings were grown for 7 days in a greenhouse (50 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) or in weak light (ca 0.1 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) in order to avoid photodestruction, before chloroplast and thylakoid isolation. NF-treatment and the low light condition also affected chlorophyll content. Isolated chloroplasts from NF-grown plants contained approximately 55 and 16 % of the chlorophyll content as compared to untreated plants grown in weak light or in greenhouse light, respectively.

In addition, protein separation by polyacryl amide electrophoresis (SDS-PAGE) on isolated thylakoids showed that the apoproteins of the LHC II (i.e. LHCP) were undetectable after NF-treatment. Despite a large number of attempts to measure PS II activity in isolated thylakoids, this was proven to give unreliable data and these were therefore excluded from this report. The photosynthetic assays were therefore focused on PS I activity, which were compared between plants grown in greenhouse (untreated) or in weak light, with or without NF. As expected, PSI activity is greatly affected by light intensity, during growth as well as during the photosynthetic assays. In untreated wheat, grown in the greenhouse, the highest activity was found at the strongest light intensity used at distance of 0.25 m from the light source (corresponding to approximately 1440\(\mu\)mol m\(^{-2}\)s\(^{-1}\)). The PS I activity then declines with decreasing intensity. A similar, albeit not so clear (lower maximum activity at strong light intensities), trend is observed in untreated wheat grown in weak light.

Thylakoids isolated from NF-treated plants show a clear photoinhibition at strong light but a clear increase in activity at subsequently lower light intensities.

Abbreviations

NF-Norflurazon, PSII-photosystem two in the photosynthesis light reaction, PSI-photosystem one in the photosynthesis light reaction, LHCII-light harvesting complex two, Cab- the nuclear gene family encoding several chlorophyll-binding proteins, kDa-kilo Dalton.
1 Introduction

The process of photosynthesis occurs in eukaryotes, plants and green algae, and in the prokaryotes, such as cyanobacteria, green and purple bacteria. The photosynthesis is the process in which light energy is converted to chemical energy which is further utilised to synthesise carbohydrates. In the photosynthetic eukaryotes photosynthesis take place in the chloroplasts. The chloroplast is surrounded by a double membrane known as the envelope. In addition, an inner membrane system, the thylakoids are embedded in an aqueous phase, the stroma (Figure 1, Raven, Evert and Eichorn, 2005). The so called light reactions which take place in the thylakoid membrane involves two separate photosystem, photosystem II (PS II), and photosystem I (PS I). Light triggers a charge separation in the thylakoids which eventually leads to an electron transfer from PS II to PSI and a reduction of NADP⁺ on the stromal side of the membrane. In the process, oxygen is released during the oxidation of water in PS II and ADP is phosphorylated to ATP.

![Fig.1. The chloroplast. (Raven, Evert and Eichorn, 2005)](image)

Granum are the participated thylakoid stacks connected by stroma lamellae. These stacks are the results of appressions between thylakoid membrane regions where the Columbic repulsive forces between two adjacent membranes are decreased (Dahlin, 2003). In the chloroplast the stroma that surrounds the thylakoids contains enzymes that catalyse carbon fixation and other biosynthetic processes. Thylakoids inner space is known as the lumen. Thylakoid membrane is the lipid bilayer with the embedded proteins which build the four complexes known as supracomplexes. As mentioned earlier the two main photosystems participate in photosynthetic light reaction, are photosystem I (PSI) and photosystem II (PSII) (Figure 2). These two large membrane protein complexes consist of antennae complexes and reaction centers. The antennae complex is a three helical transmembrane protein. The most abundant antenna proteins are light harvesting complex II chlorophyll a/b binding proteins. LHClI proteins has not yet been sequenced but supposedly they are similar to that of the LHCII (Taiz and Zeiger, 2002).
Fig.2. The four protein supra complexes in the thylakoid membrane and electron flow from H₂O to NADP⁺. Functional relationship among PSII, cytochrome b₆-f complex, PSI and the ATP synthetase (Taiz and Zeiger, 2002).

Both antenna and reaction center proteins are non-covalently associated with chlorophylls. It is supposed that all LHC proteins contain three carotenoid binding sites, which show preferential binding for the xanthophylls lutein, violaxanthin and neoxanthin (Guseinova et al., 2004).

Two polypeptides with molecular weight near 33 kD and 31 kD are commonly called D1 and D2 which directly bind P680 and certain quinones necessary for oxidation of water. The main proteins of the LHCII are the apoproteins, with molecular weights of 27 and 29 kD. Actually photosystem II provides whole photosynthetic process with electrons by extracting them from water (Frome et al., 2006).

Chlorophylls are the pigments which through light absorption promote the electron transport during photosynthesis. Chlorophyll b is not directly involved in the photosynthesis; it transfers the energy to chlorophyll a when absorbing light.

Carotenoids, as integral components of the photosynthetic apparatus have multiple roles. They are light-harvesting pigments and transfer light energy. Carotenoids have a protective role to protect the chlorophyll against photodestruction by quenching chlorophyll triplet state, in light. In addition, they maintain the function of reaction centres and light harvesting complexes of the photosynthesis, scavenging singlet oxygen species and dissipating excess energy absorbed by antenna chlorophyll. Their structural role is stabilising the light-harvesting complex (LHCII) chlorophyll a/b protein and its incorporation into the membranes. (Bolychevsteva et al., 1995 and Dahlin, 1989). Carotenoids are synthesised in envelope and localizes to the thylakoids of plastids. β-carotene is mainly localised in the core complex of PSI and PSII.

The xanthophylls violaxanthin and neoxanthin as well as lutein are predominantly found in light-harvesting complexes. Lutein participates in oligomerization of light harvesting complexes during assembly of the photosynthetic apparatus (Jung et al., 1999).
Most of the carotenoids (both β-carotene and the xanthophylls) in the thylakoids efficiently transfer their excitation energy to the same reaction centres as do chlorophylls, and so they contribute to photosynthesis (Salisbury and Ross, 1992).

Plastids are semi-autonomous organelles and contain their own DNA. Most of plastid specific proteins are encoded by nuclear DNA synthesised in the cytosol to be later transported to the plastid. Proplastids differentiate into chloroplasts during leaf development in the light. During this time the plastids accumulate thylakoid membrane proteins involved in the light reactions of photosynthesis (Dahlin and Cline, 1991).

Pigment mutants or plants treated with sub lethal concentrations of bleaching herbicides have been used to understand the correlation of pigment deficiencies to structural or functional aberrations in the chloroplasts. NF, is an herbicide, which affects chloroplasts development through interaction with components either inside or outside the plastid. This herbicide inhibits the enzyme phytoene desaturase, coded by the nuclear genome, and thereby blocks the biosynthesis of carotenoids. The absence of carotenoids results in photo-oxidation of chlorophyll, bleaching and inactivation of the photosynthetic apparatus under high, but not under low light conditions. The chloroplasts of plants treated by NF grown under high light fluency are damaged, fotosynthetically unfunctional, lack thylakoids but maintain some altered membranes.

The main effect of NF on plastids is a substantial reduction of chlorophyll-binding proteins of PSII, LHCP, and an insertion into the membrane (Dahlin and Timko, 1994; Yurina et al., 2001; Mayfield et al., 1986; La Rocca et al., 2004 and Figure 6).

The bleaching herbicide NF inhibits the long- chain carotenoids which results in a significant decrease in PSII core complexes and the content of the light harvesting (LHC) proteins and inhibition of PSII activity.

Treatment with 28 mg l⁻¹ NF, which inhibits carotenoid biosynthesis, prevented formation of appressed thylakoids, markedly interfered with accumulation of apo-LHC.

The aim of this investigation was to correlate the young wheat plants with four different treatments on background of three questions:

1. Is the chlorophyll content drastically reduced after NF treatment of the wheat plants grown under weak light and control untreated plants growing under the same condition compared with those grown in the greenhouse.
2. Is photosynthesis occurring in the wheat seedlings treated by NF and how is it affected of light by distance in relation with other two treatments.
3. Are the PSII LHC Chl a/b binding proteins absent in the isolated thylakoids from young wheat plants after treatment by NF.

The bleaching herbicide NF inhibits the long- chain carotenoids which results in a significant decrease in PSII core complexes and the content of the light harvesting (LHC) proteins and inhibition of PSII activity.
2 Materials and methods

2.1 Growth conditions
Wheat seeds (Triticum aestivum L.) were soaked for approximately 17 hours in tap water or in tap water containing 28mg l\(^{-1}\) NF, the most soluble amount of herbicide in one litre of water, (obtained from Sandoz Ltd. Basel, Switzerland) and then planted in vermiculite. The seedlings were grown in white light 50\(\mu\)mol m\(^{-2}\) s\(^{-1}\), one set, and weak light 0,1 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), one set as control and one as NF treated. All plants were cultivated in a growth chamber at approximately 20° C for 7 days. The plants were watered on the day they were planted and one more time in the middle of their growth period.

2.2 Chloroplast and thylakoid isolation

Intact chloroplasts were isolated according to this method:

Equal amounts of Percoll medium and 2X medium A (0,3 M sorbitol, 1 mM MgCl\(_2\) x 6H\(_2\)O , 2 mM EDTA, 10 mM KCl, 25 mM MES ) were blended and centrifuged at 19500 g for 20min (JA 25,50 rotor, Beckman J-25 centrifuge) to obtain a density gradient. The chloroplast isolation was performed as described below in thylakoid isolation. The isolated chloroplasts were washed by adding carefully to Percoll gradient and centrifuged at 7200 g for 15 min (JS 13,1 rotor, Beckman J-25 centrifuge). The purified intact chloroplasts were removed from the Percoll and washed twice in medium A (1X) at 3000 g for 7min (JS 13,1 rotor, Beckman J-25 centrifuge). Chloroplast concentration was estimated in a Buchner haemacytometer.

Chlorophyll pigment determination
Chlorophyll concentrations where calculated according Lichtenthaler and Wellburn (1983).

Thylakoid isolation

Approximately 30 g of the wheat leaves were cut in one cm long pieces, homogenized in mixer adding 100 ml ice-cold grinding medium A containing 0,3 M sorbitol, 1 mM MgCl\(_2\) x 6H\(_2\)O , 2 mM EDTA, 10 mM KCl and 25 mM MES, (pH 6,1-KOH). The homogenate was filtered through two layers nylon cloth and one layer of cheese cloth, and the filtrate centrifuged at 3000 g for 10 min, on 4°C (JA-10 rotor, Beckman J-25 centrifuge). The isolated chloroplasts were resuspended in medium B (10 mM MgCl\(_2\), 30 mM KCl, 5 mM MES, without EDTA and the sorbitol osmoticum and centrifuged at 3500 g for 10 min (JA-25, 50, Beckman J-25 centrifuge). The pellet (thylakoids) was resuspended in medium C, the same contents as medium B add 0, 3 M sorbitol and centrifuged at 3000 g for 10 min (JA-25,50, Beckman J-25 centrifuge ). Absorption spectra of the extracts in 1.5 ml acetone were recorded between 400 and 700 with a Helix \(\alpha\) Thermo spectronic spectrophotometer.
To avoid photodestruction in NF-treated plants thylakoid isolation was performed in dim light.
2.3 **PSI-activity**

Photosystem-I activity in isolated thylakoid membranes, corresponding to 100 µg chlorophyll/ml, was measured in a medium containing 50 mM sorbitol, 10 mM KCl, 5 mM MgCl$_2$·6H$_2$O, 1 mM EDTA, 1 mM HEPES, KOH pH 7.6, by adding 10 mM methylviologen (MV), 50 mM 3-(3,4-dichlorophenyl)-1,1dimethylurea (DCMU), and the artificial electron-donor system containing 50 mM 2,6-dichlorophenolindophenol (DCPIP) and 50 mM ascorbate. Light was provided with a Kinderman slide projector. Constant temperature was monitored by a cooling water system at 20°C.

2.4 **PSII-activity**

Photosystem-II activity was measured generally as described above for the photosystem-I activity, except that methylviologen was replaced by 100 mM phenyl-p-quinone (PPQ) and 50 mM 3-(3, 4-dichlorophenyl)-1,1 dimethylurea (DCMU).

2.5 **SDS-PAGE**

Isolated thylakoids were suspended in an equal volume of denaturising buffer (2X) and heated in a water bath at approximately 80°C for 5 min. Denatured proteins were separated by SDS-polyacrylamide gel electrophoresis 5% stacking gel and 12% separating gel. The electrophoresis was performed at 200 V for 1.5 h by using buffer system of Laemmli (1970). Thylakoid membranes, corresponding to 10 µg chlorophyll/lane, were loaded in lanes A, B and C. In lanes E, F and G, thylakoid membranes corresponding to 0, 5 µg chlorophyll/ lane were loaded. For reference, 6µl and 5µl standard proteins were loaded in lane D and H, respectively. Gels were Coomassie Blue stained and de stained in 25% (v/v) methanol and 7% (v/v) acetic acid.
3 Results

3.1 Chlorophyll/plastid

The wheat plants grown in the different cultivation conditions displayed different morphologies. Plants grown in the greenhouse had green leaves (Figure 4). Untreated plants grown in the weak light became green-yellow while NF treated plants grown in the same conditions were pale green (Figure 3). NF treated plants grown in the greenhouse became completely white (Figure 4) and contained no pigments (Table 1). Therefore, these plants were excluded from further analysis.

![Fig.3. Seven day old wheat plants, NF treated and untreated grown under weak light.](image)

![Fig.4. The NF treated plants and untreated grown in the greenhouse, seven days old.](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chl.conc.- Pg/plastid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greenhouse (not treated, )</td>
<td>0,62pg/pl</td>
</tr>
<tr>
<td>Control (not treated, weak light)</td>
<td>0,18pg/pl</td>
</tr>
<tr>
<td>NF ( treated, weak light)</td>
<td>0,10pg/pl</td>
</tr>
<tr>
<td>NF ( treated, greenhouse)</td>
<td>below detection</td>
</tr>
</tbody>
</table>

Table 1. Chlorophyll content per chloroplast. Intact chloroplasts were isolated and assayed for chlorophyll content as described in material and methods.
3.2 Photosystem I activity:

The measurements of PSI were done on purified thylakoid samples of untreated seedlings grown in the greenhouse light and in weak light, NF treated and untreated. The assays were performed on one week old plants at different distances from the light source. The oxygen consumption showed different values with distances from the light source. As seen in figure 5, the light intensity during the assay had very different effects on the photosynthetic activity in PSI depending on herbicide treatment and growing conditions.

**Oxygen consumption in PSI**

Fig.5. The photosynthetic PSI activity in isolated tylakoids. Greenhouse, untreated plants grown in white light; Control, untreated plants grown in weak light; and finally NF, plants treated with Norflurazon grown in weak light. The values in meters indicate distance from the light source.

The weak light enables the NF treated plants to accumulate low amounts of chlorophyll. The oxygen consumption shows different values in connection with distance from light.
3.3 SDS-PAGE

During the absence of stabilising pigments, carotenoids and chlorophylls, caused by NF treatment of wheat plants, PSII protein complex become decomposed. The isolated LHCII chlorophyll a/b binding proteins 29 kD polypeptides are depleted and not detected among the thylakoid polypeptides isolated from plants treated with 28 mg l⁻¹ NF (Figure 6).

![Fig. 6. Elektrophoretoogram of thylakoid proteins. The thylakoids were isolated from wheat seedlings grown in a greenhouse (A) and (E) or in weak light (B, C, F and G) for seven days. Slots D and H indicate the standard proteins whereas lanes (C) and (G) indicate the thylakoids isolated from NF treated plants. The lanes were loaded with 1 µg/µl (E-G) and 0.5 µg/µl (A-C) per slot samples and 6µl and 5 µl standard proteins (D and H, respectively). The gel was Coomassie Blue stained. Positions of standard proteins are given. MW, molecular weight in kD (kilo Dalton). Reference proteins are phosphorylase b (97,400), serum albumin (66,200), ovalbumin (42,700), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400).]
4 Discussion

The plants treated with 28 mg l⁻¹ NF grown in the greenhouse became thoroughly photo bleached. The high light intensities cause bleaching of the leaves in the absence of the carotenoids. The carotenoid content in these plants are below that needed for photoprotection. Chlorophyll bleaching did not appear in carotenoid deficient seedlings illuminated with weak light.

Generally plants treated with NF grown in darkness accumulate the colourless carotenoid precursor, phytoene and phytofluene, while devoid of the coloured carotenoids lutein and β-carotene (Jung et al., 1999). Hence the NF inhibits the enzyme phytoene desaturase which blocks biosynthesis of carotenoids in plant (Nadezda et al., 2001). As a consequence of the absence of carotenoid, plants also accumulate low levels of chlorophylls compared to untreated plants grown under the same conditions (Dahlin and Cline, 1999; Gorton et al., 1980 and Table 1). The photo destructive effects were low or not at all observed in the NF treated wheat plants grown in the weak light (Dahlin, 1989 and Figure 3). In the leaves grown in the dark with carotenoid deficiency the formation of long wavelength protochlorophylide is still induced (Yahubyan et al., 2001).

Most of the plastid proteins are encoded by nuclear genes and synthesised in the cytosol to be later imported in to the organelle. Expression of the genes coding for PSII polypeptides are not affected by NF in weak light which is also the case for nuclear mRNA. The indications that although mRNA levels of Cab of the light-harvesting complex of PSII between control and NF treated plastids grown in the weak light were similar, the amount of the proteins was reduced (Sagar and Briggs, 1990) or absent (Dahlin, 1989 and Figure 6) were reasons to the further investigations. The recent studies suggest that NF treated plastids, with carotenoid deficiency, are able to bind precursor proteins on the surface but are unable to import the proteins across the envelope membranes which explains the role of carotenoids on the stabilising of the plastid import apparatus (Dahlin and Franzén, 1997).

Chloroplasts of plants treated with NF grown in the greenhouse are totally devoid of thylakoids (La Rocca et al., 2004) whereas those grown in the weak light consisted of stroma thylakoids lacking appressed regions (Dahlin, 1988 and Dahlin, 2003). According to Mayfield et al., (1986) plastids developed in carotenoid-deficient seedlings in the low light intensity accumulate normal LHC II mRNA levels. The effect of carotenoid deficiency is expressed on pretranscription when plastids are still young. The LHC II is necessary for the formation of thylakoid appressions in chlorophyll b-containing plants. In wheat treated with NF and grown under weak light the proportion of thylakoid appressions correlated with the degree of presence of LHC II proteins (Dahlin, 1989). No thylakoid appressions are present in the plastids of plants treated with 28 mg l⁻¹ NF. Wheat treated with 28 mg l⁻¹ NF grown under weak red light contain markedly lower proportion of apo-LHC then un treated plants grown under the same light conditions.

Nonappressed regions of grana and stroma thylakoids have far less PSII (Taiz and Zeiger, 2002 and Raven, Evert and Eichorn, 2005). The sensitivity of the photoreceptor and response systems is unaltered by the herbicide. (Gorton et al., 1980). There was no difference in the responsiveness of treated and untreated plants.

The measurements of PSII and PSI were done on purified thylakoids from untreated seedlings grown under normal white light and weak light, NF treated and untreated respectively. The assays were performed on one week old plants at different distances every week. The membranes isolated from plants grown under weak light in control and after treatment exhibited more fluctuated values of PSII (results are not shown). As expected, there were no oxygen evolution during assays with NF treated plants.
In addition PSI values of greenhouse grown plants show the linear decrease on first three distances from light. At next distance from light exhibits the drop of PSI activity with further decreasing by distance which leads to the suggestion that it is due to decreasing light intensity by square of distance and that light intensity is less considerable after every distance. PSI activity of untreated plants grown in the weak light has shown irregular decreasing after which came the rise of rate. This explains as ability of plants grown in the weak light to build more light capturing antennas during growth period as a consequence of limited light. Lower PSI values, at shorter distance from light, of NF treated plants, can actually be explained by photodestruction greenhouse light on thylakoids without protection in the carotenoid deficient plants.

The polypeptide composition of isolated plastid tylakoids of untreated plants grown in the greenhouse and in weak light with or without the herbicide NF analysed by SDS-PAGE show that the LHC II chlorophyll a/b binding protein is absent in the samples of NF treated plants (Figure 6). Chlorophyll content in the chloroplasts of untreated plants grown in weak light was ca 29% of those grown in greenhouse (Table 1). The chlorophyll content of NF treated plants grown in the light of low intensity compared with that of untreated grown in the white light was 16%. NF treated plants grown in the weak light contained 55% of the chlorophyll content compared to plants grown under the same condition (Table 1). Furthermore carotenoid content in the plants treated with the herbicide NF is approximately 0,5 and 2,0% compared with the normal carotenoid content in etioplasts and chloroplasts of wheat seedlings (Dahlin, 1989). The conclusion can be that the chlorophylls and carotenoids in the NF treated plants grown in the weak light are significantly lower than in the untreated plants grown under the same light conditions and in the greenhouse (Dahlin, 1994 and Table 1).

Many assays are done but still there are much to investigate about the role of carotenoids and other plastid components cooperation and NF influence on their ultrastructure and functions. These investigations can be useful for further research concerning carotenoids and their role in photosynthesis.

**Acknowledgement**

I would first of all like to thank my supervisor Clas Dahlin for his undoubted support, guidance and going through manuscript. I would like to acknowledge all personal I asked for chemicals and other material during examinations performance. Also my schoolmates for suggestion and moral support will me to thank for.
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Appendix

Isolation medium A for chloroplasts (250 ml)

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<th>Konc</th>
<th>Mr</th>
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<tr>
<td>1mM</td>
<td>MgCl₂ x 6H₂O</td>
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<tr>
<td>2mM</td>
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<tr>
<td>10mM</td>
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<tr>
<td>25mM</td>
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pH 6.1 with KOH

ad before using:

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<td>2mM</td>
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Isolation medium B (100 ml)

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<td>5mM</td>
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pH 6.5 med KOH

Isolation medium C (100 ml)

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pH 6.5 med KOH

Reaction medium (500 ml)

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pH 7.6 med KOH

Reagens and inhibitors:

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<td>CCCP (in etanol)</td>
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