The Effect of Phosphinothricin (Glufosinate) on Photosynthesis
II. The Causes of Inhibition of Photosynthesis

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Z. Naturforsch. 42c, 270–278 (1987); received September 12, 1986

Ammonia, Chloroplasts, Glufosinate, Glutamine, Glutamine Synthetase

It was shown in the previous study that phosphinothricin (glufosinate) causes an accumulation of ammonia and inhibition of photosynthesis. The extent to which there is a connection between these two processes is now investigated in the present study. First of all, the role of NH₃ per se in the impairment of photosynthesis was to be clarified. For this purpose, the inhibition of photosynthesis was investigated in relation to exogenously applied ammonia in chloroplasts, protoplasts and entire leaves. The comparison with the experimental results in leaves in which the ammonia was formed endogenously (by action of phosphinothricin) shows that the ammonia toxicity at least cannot be solely responsible for the impairment of photosynthesis. The inhibition of photosynthesis is maintained under non-photorespiratory conditions, although considerable amounts of NH₃ are concentrated at the same time in the plants. A process connected with photorespiration appears to play the essential role.

Simultaneous administration of phosphinothricin and glutamine largely prevented the impairment of photosynthesis. This indicates that the main cause for the inhibition of photosynthesis by phosphinothricin is based on a depletion of glutamine. Three subsequent reactions which may cause the inhibition of photosynthesis are discussed: 1) an inhibition of protein biosynthesis, 2) a toxic accumulation of glyoxylate in the photorespiratory cycle and 3) a deficiency of intermediates of the Calvin cycle in consequence of the interrupted photorespiration.

Introduction

In earlier studies, we were able to show that the treatment of plants with PPT brings about an irreversible inhibition of glutamine synthetase [1]. In consequence of this enzyme inhibition, there is a rapid accumulation of NH₃ in the leaves and a reduction of photosynthesis [2, 3]. The major accumulation of ammonia may suggest that NH₃ per se is responsible for the impairment of photosynthesis, as also postulated by various authors for the treatment with MSO [4–9].

Although NH₃ is an entirely physiological substance (10), large amounts are toxic to plants [5, 6, 11]. Ammonia(um) interferes with metabolism at various sites. So far, six mechanisms of cell damage have been postulated: 1) the uncoupling of photophosphorylation [12–14]. The toxic concentration have been reported to be 0.6 mM [10], 2 mM [11] and even 5–10 mM [15]; 2) the confusion of NH₃ with K⁺ – the increased ammonium concentration in the cells caused by administration of PPT correlates with the higher K⁺ permeability [16, 17]; 3) the inhibition of the photodependent activation of ATP synthetase [18]; 4) the inhibition of NADP⁺ reduction [19]; 5) the inhibition of chlorophyll biogenesis [20, 21] and finally 6), the inhibition of cell division [22].

However, the finding that photosynthesis is largely maintained under non-photorespiratory conditions although considerable amounts of NH₃ are concentrated simultaneously in the leaves suggests that ammonia toxicity at least cannot be responsible on its own for the inhibition of photosynthesis. A process connected with photorespiration probably plays a major role. In order to explore more precisely the causes of the inhibition of photosynthesis in PPT treatment, i.e. the connection between the inhibition of GS on the one hand and the reduction of CO₂ fixation on the other hand, we therefore carried out further investigations: exogenous ammonia was added to protoplasts, chloroplasts and entire leaves.

Abbreviations: BSA, bovine serum albumin; EDTA, ethylenedinitrilotetraacetic acid disodium salt; Glu, glutamate; HEPES, N-2-hydroxyethyl-piperazine-N-ethane sulfonic acid; MES, 2-(N-morpholino)-ethane sulfonic acid; MSO, L-methionine sulfoximine; OAA, oxaloacetate; OG, 2-oxoglutarate; P₃, orthophosphate; PP₃, inorganic pyrophosphate; PPT, δ-l-phosphinothricin (glufosinate); PVP, polyvinylpyrrolidone.
The correlation between inhibition of photosynthesis and NH$_3$ content could then be compared with the experiments in leaves in which the ammonia was generated endogenously by the action of PPT. In addition, the role of glutamine in connection with the effect of PPT was also investigated.

**Material and Methods**

**Plant material and chemicals**

Plants of *Valerianella locusta* (= *Val. olitoria*) and *Sinapis alba* were grown as in [2], except that the photosynthetically active radiation for *Valerianella* was about 60 W m$^{-2}$.

DL-Homoalanin-4-yl(methyl)phosphinic acid (PPT, glufosinate, code no. Hoe 035956) was supplied by Hoechst AG (Frankfurt/M., West Germany), see [3].

**Measurements of photosynthesis**

CO$_2$-fixation was determined with the method previously described [23], experiments were conducted with the primary leaves. Oxygen evolution of chloroplasts and protoplasts was measured with an O$_2$-electrode, for the illumination of chloroplasts a red filter was used (RG 610, Schott).

**Isolation of protoplasts and chloroplasts**

Protoplasts and chloroplasts were isolated by enzymic digestion, the isolation was approximately as described in [24]. Because the photosynthetic activity of the parent tissue is very important for yielding chloroplasts and protoplasts with high rates of photosynthesis [24], first of all leaves of *Valerianella* were examined with an infrared gas analyser. The experiments indicated that the best point of harvesting was about 21 days after sowing.

For preparing leaf tissue for digestion, the epidermis was removed from the lower surface of the leaf. Leaf tissue was put in a 9 cm diameter petri dish with 10 ml of the enzyme medium (I) containing: 0.5 m sorbitol, 5 mm MES, 1 mm CaCl$_2$ and furthermore 0.5% (w/v) cellulase (Onuzuka R 10), 0.5% (w/v) pectinase (Macerocym R 10), 0.1% (w/v) BSA, 0.05% (w/v) PVP; pH 5.5 (HCl).

Following incubation for 1½ h at 28 °C, the isolation medium was gently removed (using a pasteur pipette) and discarded. Three times washing with 10 ml of a medium (II) with 0.5 m sorbitol, 5 mm MES, 1 mm CaCl$_2$; pH 6.0 (KOH) set the chloroplasts free of the tissue. Undigested tissue was removed by filtration through 500 μm and 200 μm nylon meshes. The extract was centrifuged for 1 min and −2 °C at 95 × g, and the pellet was resuspended in a medium (III) containing 0.5 m sorbitol, 30 mm HEPES, 2 mm NaHCO$_3$, 1 mm CaCl$_2$; pH 7.6 (KOH). A further purification by flotation in a step-gradient was not necessary, but for removing isolated chloroplasts which were present due to rupture of protoplasts, the suspension was stored on ice for 25 min, while the protoplasts sedimented. The supernatant was discarded and the pellet resuspended in 1–2 ml of medium III. The protoplasts were stored on ice.

At all steps of the isolation glassware and solutions were kept chilling.

For the isolation of chloroplasts the protoplasts were added 1–2 ml assay medium (IV) containing 0.33 m sorbitol, 50 mm HEPES, 10 mm NaHCO$_3$, 2 mm EDTA, 1 mm MgCl$_2$, 1 mm MnCl$_2$; pH 7.6 (KOH). Then, the protoplasts were passed twice through a 20 μm nylon mesh. The extract was centrifuged for 1 min at 0 °C and 180 × g; after discarding the supernatant the pellet was resuspended in 1–2 ml of medium IV.

**Intactness assay**

The percentage of intact chloroplasts was determined from rates of ferricyanide-dependent oxygen evolution before and after osmotic shock [25].

**Determination of chlorophyll and ammonia**

Chlorophyll of chloroplasts and protoplasts was extracted in 80% acetone and measured as described in [26]; ammonia was determined as in [3].

**Results**

**Effect of exogenous NH$_3$ on photosynthesis by protoplasts**

Under the microscope, the isolated protoplasts had a regular, round shape; the chloroplasts were arranged close to the plasma membrane. Even five hours after the isolation, they were fully active in photosynthesis. The rates specified here are generally mean values of two to three, and sometimes also four light/dark phases. The variations were relatively slight. Over a period of 35–45 min (=4–5 light/dark phases), the standard deviations were less than 5%.
The initial rates of photosynthesis average e.g. in the curve of Fig. 1A 124 μmol O₂ mg⁻¹ Chl h⁻¹ (mean value of 55 measurements); the highest rate of O₂ evolution was 174 μmol O₂ mg⁻¹ Chl h⁻¹.

The effect of ammonia on the O₂ evolution in protoplasts was tested as follows: After a dark phase, the protoplasts were illuminated until a constant rate of photosynthesis had become established. The O₂ evolution markedly decreased after addition of NH₄Cl compared to the control. On the other hand, only a slight alteration in the respiration value measured in the subsequent dark phase could be detected. There were different effects when ammonia was added in the light or during a dark phase. The spontaneous consequence of the addition in light is indeed initially a raised O₂ release. However, afterwards the evolution of O₂ is appreciably reduced. On the other hand, addition of NH₄Cl in darkness initially brings about a short-term elevation of O₂ consumption. A constant uptake or evolution of O₂ is only established after the subsequent illumination phase.

In accordance with these experiments, Fig. 1 shows two different experimental series with protoplasts of Valerianella: in the one series NH₄Cl was added in darkness (1A). In the other series the application was carried out in light (1B). The unit plotted in percent in the graphs relates to the photosynthetic activity still taking place in the subsequent illumination phases.

In light, different pH conditions prevail in the organelles than in darkness. Indeed, a somewhat different course of the curve could be observed: whereas after addition of NH₄Cl in darkness the rate of photosynthesis was already inhibited by 1 mM NH₄Cl, this concentration of ammonia given in light indeed proved to promote photosynthesis. On the other hand, large amounts of NH₃ had somewhat greater inhibitory effect in light than in darkness.

The ammonia content at the end of the experiments was determined in some experiments. In the low concentrations (1–6 mM NH₃), a reduced NH₃ concentration in relation to the ammonia initially given was always recovered (about 35% less NH₃). This is assumed to be caused at least partly by an assimilation of ammonia taken up externally be means of the GOGAT cycle.

Furthermore, it was investigated as to wether the protoplasts accumulate ammonia. In order to be able to differentiate between the NH₃ concentration localized internally in the cells and the NH₃ concentration present externally in the ambient medium, we sedimented the protoplasts. The assay revealed that more ammonia was present per unit of volume in the pellet than in the supernatant.

In order to justify the comparison between the protoplast isolations with Valerianella and the measurements of gas exchange with leaves of Sinapis as the experimental object, some experiments on the
O₂-electrode were also carried out with Sinapis protoplasts. The percentage inhibition of photosynthesis by ammonia was in the same range as in the Valerianella cells.

Effect of exogenous NH₃ on photosynthesis by chloroplasts

The chloroplasts were isolated from the protoplasts. The degree of intactness measured on the oxygen electrode showed values between 74% and 100%. (With uncoupling, the thylakoids showed rates of about 180 μmol O₂ mg⁻¹ Chl h⁻¹. However, their photosynthetic activity fell rapidly even after a short time.)

For an optimal photosynthetic activity, the chloroplasts with an intact envelope (class A) prepared here required a series of reagents which had to be added exogenously: — PPT, its optimum concentration was determined to be 4 mM; — Pₙ, the optimum concentration was astonishingly high (3 mM); — PGA, its influence on photosynthetic activity revealed a saturation curve, the concentration of 3 mM was used; — in addition, 5 mM oxaloacetate, 1 mM ATP and 1 mM ADP.

With regard to time-dependence the chloroplasts were very much more sensitive than the protoplasts. Even a few hours after the isolation, their activity markedly decreased. In illumination with light/dark phases, the organelles showed a relative constant rate of photosynthesis from about the 12th to the 24th minute. Within this period, there was then inhibition by ammonia so that no overlarge fluctuations due to the aging of the chloroplasts could falsify the picture of NH₃ toxicity.

Fig. 1C shows the rates of photosynthesis in the chloroplasts in relation to the exogenously added ammonia concentration (the ammonia was added in the dark). It is interesting that the inhibition of chloroplasts resembles the protoplasts treated in light (1B): the rate of photosynthesis initially rose at low ammonia concentrations. However, this behavior was still far more pronounced in the chloroplasts, in which the O₂ evolution was raised by an average of 26% on addition of 1 mM ammonia. It was still stimulated by 4% even at an NH₃ content of 2 mM. In addition, a long lag phase of photosynthesis after the addition of NH₃ (not shown) was striking in these experiments; this could already be observed in Pisum sativum chloroplasts [14].

Effect of PPT and Gln on photosynthesis by intact leaves

It was shown in previous experiments with PPT treated leaves that photosynthesis was totally inhibited under photorespiratory conditions (21% O₂) while under non-photorespiratory conditions (2% O₂) the photosynthetic activity was largely maintained, although considerable amounts of NH₃ were concentrated at the same time in the plants. In order to study the influence of Gln on the inhibition of photosynthesis by PPT, experiments were carried out in which the leaves were supplied with PPT and 50 mM Gln via the petiole. Fig. 2 shows the results compared to those of PPT treated leaves. It was found that the addition of Gln stimulated the accumulation of NH₃. After 1 h with PPT + Gln 46 ± 9 μmol NH₃ g⁻¹ fr. wt. and with PPT alone 31 ± 6 μmol NH₃ g⁻¹ fr. wt. were generated. Already the control plants supplied with 50 mM Gln showed more than the double content of NH₃ compared to those plants that were only treated by water (12 ± 4 and 5 ± 0.5 μmol NH₃ g⁻¹ fr. wt. respectively). After the application with PPT and Gln remarkable amounts of ammonia were accumulated. Nevertheless the CO₂ fixation was largely maintained. The absolute initial rates were 45 ± 5 mmol CO₂ m⁻² h⁻¹ (treated) and 41 ± 4 mmol CO₂ m⁻² h⁻¹ (control).

Effect of NH₃ on photosynthesis by intact leaves

Fig. 3 shows a series of experiments in which NH₃ was exogenously supplied to the leaves via the petiole. The concentrations applied were 40 and 60 mM. At the end of the experiments the NH₃ content of the leaves was determined. The initial rate of photosynthesis was 44 ± 5 mmol CO₂ m⁻² h⁻¹. The resulting graph was very similar to the curve obtained with PPT + Gln treated leaves. In plants, treated with PPT alone, photosynthesis was totally inhibited at simultaneous NH₃ concentrations of 30–40 μmol g⁻¹ fresh weight. The additional treatment with Gln kept the photosynthetic activity — in the same range of NH₃ concentration — to 70% of the control. In plants supplied with exogenous NH₃, photosynthesis was maintained to 85% at this NH₃ content.

In Table I the inhibition of photosynthesis at a simultaneous ammonia concentration of 30 mM is summarized for the different experimental series with protoplasts, chloroplasts and intact leaves.
Fig. 2. Photosynthetic activity (---) and ammonia accumulation (—) of mustard primary leaves treated with PPT (1 mM), PPT/Gln (1 mM/50 mM) and H₂O (control). Measuring occurred under photorespiratory conditions (21% O₂, 400 ppm CO₂). The 100% value of photosynthesis was calculated from the photosynthetic rate at the beginning of the experiment. PPT and PPT/Gln, respectively, was added at zero time. For PPT/Gln, photosynthesis was plotted as % of control values (Gln alone). Ammonia concentration was measured at the end of the experiments. Means from 10 experiments (treated) and 4 experiments (control), respectively. The single values of the measurements are omitted for the sake of clearness.

Fig. 3. Relationship between photosynthesis and ammonia concentration after different treatment of mustard primary leaves (petiolar feeding). Measuring occurred at 21% O₂ and 400 ppm CO₂: ○ = control (H₂O); ○ = PPT; ● = PPT/Gln; △ = NH₄Cl.
Table I. Photosynthetic inhibition (%) at an ammonia level of 30 mM.

<table>
<thead>
<tr>
<th>System</th>
<th>Treatment</th>
<th>Photosynthetic inhibition [%]</th>
</tr>
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<tbody>
<tr>
<td>Protoplast</td>
<td>Ammonia addition in dark, 21% O₂</td>
<td>69</td>
</tr>
<tr>
<td>Protoplast</td>
<td>Ammonia addition in light, 21% O₂</td>
<td>83</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>Ammonia addition in dark, 21% O₂</td>
<td>86</td>
</tr>
<tr>
<td>Leaf</td>
<td>PPT, 21% O₂</td>
<td>93</td>
</tr>
<tr>
<td>Leaf</td>
<td>PPT, 2% O₂</td>
<td>20</td>
</tr>
<tr>
<td>Leaf</td>
<td>PPT/Gln, 21% O₂</td>
<td>18</td>
</tr>
<tr>
<td>Leaf</td>
<td>Petiolar feeding with ammonia, 21% O₂</td>
<td>12</td>
</tr>
</tbody>
</table>

Discussion

The effect of exogenous NH₃ on chloroplasts

The effect of ammonia on photosynthesis in chloroplasts depends on its concentration (Fig. 1c). Large amounts of ammonia give rise to total uncoupling. This also results in inhibition of photosynthesis: half-maximum O₂ evolution was observed at 8–9 mM NH₃. The degree of inhibition of photosynthesis at a certain ammonia concentration evidently also differs greatly within the same plant species: some authors found with pea chloroplasts that photosynthesis at 10 mM NH₃ was only 21% of controls [8]. Other investigators observed half-maximum inhibition only at an NH₃ content of 15 mM [14]. On the other hand, small amounts of ammonia bring about an intensification of O₂ evolution. This phenomenon was already discovered in earlier studies on thylakoids, and has also been discussed [15, 27–29].

The effect of exogenous NH₃ on protoplasts

Even in protoplasts, extracellular NH₃ in high concentrations also brings about an inhibition of photosynthesis. Initially, a stimulatory effect is likewise noticeable here. If NH₃ is added in the dark, it immediately gives rise to a greater O₂ release. It is probably attributable to the uncoupling of oxidative phosphorylation which initially brings about a stimulation of mitochondrial electron transport. On the other hand, on addition of NH₃ in light, there is initially a greater O₂ release. This might possibly be due to uncoupling of photosynthesis.

In protoplasts to which NH₄Cl is added in light, exactly as in the chloroplasts low ammonia concentrations lead to a lasting increase of O₂ evolution (Fig. 1B). This is not the case when NH₄Cl is applied in darkness (Fig. 1A). It is understandable owing to the buffering action of the cytosol that the half-maximum inhibition values of photosynthesis (13 mM in the dark and 11 mM NH₃ in light) are somewhat higher than in the chloroplasts.

The role of NH₃ in inhibition of photosynthesis by PPT

These experiments with chloroplasts and protoplasts in which the exogenously added ammonia thus alone led to inhibition of photosynthesis could then be compared with experiments on leaves poisoned with PPT in which the NH₃ was endogenously accumulated by the action of PPT. At low ammonia concentrations, it is observed that the protoplasts are indeed damaged somewhat sooner than the leaves. This can be explained as has been described above in that the protoplasts evidently act as an ammonia trap.

In the region of larger amounts of ammonia, photosynthesis in the leaf already stops at NH₃ concentrations which still allow a certain amount of photosynthesis in protoplasts. However, the CO₂ fixation rates are investigated with the leaves on the URAS, whereas the experiments with protoplasts involve oxygen measurements. In this situation, the electrons are able not only to serve for CO₂ reduction, but also for ammonia assimilation. Anderson and Walker [30] were able to demonstrate an ammonium-2-oxoglutarate-dependent O₂ evolution in a magnitude of 6–11 μmol O₂ mg⁻¹ Chl h⁻¹ in a reconstituted spinach chloroplast system. For comparison, in protoplasts, the oxygen evolution is still about 23% in a NH₃-range in which the leaf photosynthesis is completely inhibited (40 mM). If an absolute rate of only 100 μmol O₂ mg⁻¹ Chl h⁻¹ is assumed (photosynthetic activities were very much higher in most cases), this would entail a CO₂-independent O₂ development of 23 μmol O₂ mg⁻¹ Chl h⁻¹. However, this value is just three times higher than that deter-
mined in [30] for ammonia assimilation. A certain percentage of the released electrons indicated by the oxygen evolution must indeed be used for photosynthesis. This finding indicates that another mechanism than NH$_3$ toxicity must be involved in the damage to photosynthesis by PPT.

This was also suggested by the experiments which were carried out under non-photorespiratory conditions [3]. If one assumes e.g. an ammonia concentration of 30 mM then it is shown that the CO$_2$ fixation in 2% O$_2$ was only reduced by 20%. On the other hand, under photorespiratory conditions only 7% of the initial rate of photosynthesis was present at this NH$_3$ content (Table I).

These results clearly show that it is not only the effect of NH$_3$ per se which is responsible for the photosynthetic damage. In addition, the fact that photosynthesis remains largely intact under non-photorespiratory conditions leads to the assumption that some process in connection with photorespiration must finally cause the toxicity of PPT.

**Toxic processes resulting from the effect of PPT**

Our investigations showed that transpiration remains largely the same in treated plants and controls, so that a PPT-induced closure of the stomata can be ruled out as the cause of the inhibition of photosynthesis. In rare cases, stomatal resistance is somewhat raised. However, as was also observed with MSO, the stomata are never entirely closed [6, 31, 32].

It is known that chemical or genetic inhibition of the photorespiration cycle is a toxic process for the plant [33]. Inhibition of glutamine synthetase as an enzyme of the photorespiratory N cycle leads on the one hand to accumulation of NH$_3$, but on the other hand also to suppression of glutamine synthesis. Via the reaction with OG to Glu, transaminations occur from this amino acid. Indeed, various authors have detected a major decrease in concentration of many amino acids after inhibition of GS by MSO [34–37].

If these Gln-dependent reactions are in fact responsible for the damage to photosynthesis by PPT, inhibition would have to be reduced when Gln is applied in addition to PPT.

**Investigations of Gln-dependent processes in inhibition of photosynthesis by PPT**

Leaves to which 1 mM PPT and 50 mM Gln were added still showed a high photosynthetic activity despite a more than three times higher ammonia concentration compared to the leaves treated with PPT alone (Fig. 2). In the range in which leaves provided with PPT are already totally damaged, photosynthesis in additional Gln administration is still roughly 70–80% of the initial rate. This proportion is thus caused by the collapse of the Gln-dependent reactions. The residual inhibition (i.e. 20–30%) is then attributable to the ammonia toxicity. The addition of Gln to the herbicide evidently has a protective effect on the plants. The exogenously added Gln probably provides the amino nitrogen for the transaminations.

A further confirmation of this notion are experiments in which exogenous NH$_3$ was applied to the leaves via the petiole (Fig. 3). Even at very high NH$_3$ concentrations, there is only a relatively small decline in photosynthesis. Just like the action of exogenous Gln, this is also in agreement with the result on plants treated with MSO [32, 36, 38]. In accordance with expectations, the inhibition curve for NH$_3$ reveals a similar course to that in plants treated with PPT + Gln: in both cases, the decrease in photosynthesis is finally attributable to the NH$_3$ toxicity alone. Ammonia, exogenously added or produced endogenously by addition of PPT and Gln thus causes a comparable inhibition of photosynthesis.

Accordingly, the lack of Gln is essentially responsible for the early damage to photosynthesis by PPT. On the one hand, transaminations are no longer possible. However, this is improbable to play a role in the rapid toxic effect of the herbicide. On the other hand, the lack of transaminations has a fatal effect on photosynthesis. The Glu required for this depends on resupply on the basis of Gln. However, Glu itself is indeed subject to a raised metabolism during inhibition of GS [32], so that it is no longer available for the transaminations. This disturbance of amino acid metabolism might act on photosynthesis in a different way (Fig. 4):

1) Inhibition of protein biosynthesis. The Q$_B$ protein involved in electron transport displays a very high, light dependent turnover [39, 40]. A lack of regeneration of these redox component would result in a collapse of electron transport.

An amino donor would not longer be present even for the transamination of glyoxylate. However, since the oxygenase reaction nevertheless occurs under photorespiratory conditions, the falling apparent rate of CO$_2$ fixation might be a result of direct CO$_2$ release from glyoxylate [41, cf. 42].
2) Toxic glyoxylate accumulation. Finally, another possibility is the accumulation of glyoxylate. It is a reversible inhibitor of RubP carboxylase/oxygenase [43]: at a concentration of 10 mM, a 50% inhibition of the enzyme already occurs after 2 min. This finding is supported by experiments on Arabidopsis mutants with damage of the serine-glyoxylate aminotransferase [44].

3) Lack of intermediates of the Calvin cycle. Lack of active GS or any other enzyme which prevents regeneration of the carbon channelled into the photorespiration cycle by the oxygenase reaction finally results in a lack of RubP for the Calvin cycle. This might also be a factor contributing to the inhibition of photosynthesis (for MSO: [34, 36]).