**Avena Coleoptile Segments:**
Hyperelongation Growth after Anaerobic Treatment

A. Hager

Institut für Biologie I, Universität Tübingen, Auf der Morgenstelle 1, D-7400 Tübingen

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_Avena sativa_ coleoptile segments show an anomalous increase in elongation growth following a short period of oxygen deprivation (tested between 0 and 60 min) lasting 20–30 min (anaerobiosis-aerobiosis transition effect = ANA effect). The increase in growth rate is 600% and is commensurate with that observable following an auxin treatment. This hyperelongation growth, in contrast to the auxin-induced growth, begins without a lag phase.

The growth "burst" following anaerobiosis is similarly to auxin-induced elongation growth, and is suppressed increasingly by neutral or more alkaline buffers.

Hyperelongation growth is suppressed by respiratory inhibitors and uncouplers. A complete inhibition is effected with KCN (0.5 mM) sodium azide (0.5 mM) and CCCP (1 μM); amytal (in the range 0.5 to 1 mM) and sodium arsenate (0.1 to 1 mM) are strong inhibitors. Some of those (e.g., KCN, arsenate, amytal) cause a slight increase of the ANA effect in very low concentrations, which is probably due to the K+ or Na+ ions present; on their own, these ions have a strong positive influence on the ANA effect.

During anaerobiosis the ATP level sinks around 75% and almost returns to the old value, following the supply of air, within one minute. The cell sap pH drops from 6.3 to 5.9 during anaerobiosis within 20 min. This lowering is mainly due to an increase in lactic acid concentration. Other acids such as citric, malic, and aspartic acids show insignificant changes in concentration.

The NADH content increases during anaerobiosis, whereas that of NADPH drops almost as much.

The mentioned changes in concentration of lactic acid, NADH and NADPH return to the control value within 20–30 min; thus the differences exist as long as hyperelongation growth is under way.

Possible relationships between the mentioned chemical changes and hyperelongation growth are discussed. One of the possible explanations is the following: the lowering of the cytoplasmic pH (normally around pH 7) during anaerobiosis due to the formation of lactic acid causes an activation of H⁺-ATPases in the plasmalemma and ER, since their optimum activity occurs in a pH range from 5.5 to 6.5. This activation causes a greater H⁺-excretion into the cell wall compartment, and thus hyperelongation growth following supply of air and of ATP.

Beispielsweise soll Avena Coleoptile Segments: Hyperelongation Growth after Anaerobic Treatment beinduziert werden. Die Autoren untersuchen das Verhalten von Avena Coleoptile Segments in anaerobem Medium und zeichnen die Wirkung von Respirationshemmern und Uncouplern auf. Es wird berichtet, dass eine vollständige Inhibition mit KCN (0.5 mM), Natriumazid (0.5 mM) und CCCP (1 μM); Amytal (in der Konzentration von 0.5 bis 1 mM) und Natriumarsenat (0.1 bis 1 mM) erreicht wird. Einige dieser Substanzen (z.B. KCN, Arsenat, Amytal) bewirken eine geringfügige Erhöhung des ANA Effekts in sehr niedrigen Konzentrationen, die wahrscheinlich auf die K⁺ oder Na⁺-Ionen zurückzuführen sind.


Der NADH-Gehalt steigt während der anaeroben Behandlung, während der NADPH-Gehalt fast abfällt. Die Unterschiede bestehen solange wie der hyperelastische Wuchs während der anaeroben Behandlung auftritt.

Es wird auch berichtet, dass die erwähnten chemischen Veränderungen mit dem hyperelastischen Wuchs in Beziehung stehen könnten. Eine Möglichkeit besteht darin, dass die Abnahme des cytoplasmatischen pH-Wertes (normalerweise um pH 7) während der anaeroben Behandlung zu einer Zunahme der Laktatsäure führt, die eine Aktivierung der H⁺-ATPase in der Plasmalemma und ER bewirkt, da deren Optimalaktivität in einem pH-Bereich zwischen 5.5 und 6.5 liegt. Dieser Aktivierung wird die stärkere H⁺-Exkretion in die Zellwandfaser zugeschrieben, was die hyperelastische Wuchsreaktion auf die Zufuhr von Sauerstoff und ATP erklärt.

**Introduction**

Coleoptile segments show a “burst” of growth, lasting 20–30 min, after being subjected to anaerobic conditions [1]. The rate of growth is up to six times that preceding the period of oxygen deprivation. In spite of the inhibition of growth during anoxia, the coleoptile has a greater absolute length after the growth "burst" than the control constantly immersed in an aerated solution. This so-called ANA effect (anaerobiosis-aerobiosis-transition effect) can be induced in coleoptile segments no longer in contact with the auxin-producing tip. In contrast to auxin-induced growth rate increases, which begin after lag phases of 10 to 15 min, the ANA effect is characterized by immediate hyperelongation growth when oxygen is supplied. It was therefore supposed that the accumulation of glycolysis products or changes in condition of the protoplasm during anaerobiosis could be directly related to the growth "burst" following the supply of oxygen.

Parrish and Davies [2, 3] have also observed a growth increase in dicots (pea stem segments) after anaerobic treatment, which, however, lasted several hours. They trace this intensified growth from a release of auxin from chemically or physically sequestered pools during anaerobiosis.

In this paper further characteristics of the ANA effect are described and chemical changes in cole-
optile segments during the anaerobic period are investigated. Possible causes of the growth effect are discussed.

**Materials and Methods**

The culture of *Avena sativa* ("Siegshafer I, Svalöf, Sweden") was carried out on damp cellulose at 21 °C and 90% relative humidity. Coleoptile segments 5 to 6 day old and 1.8 to 2.0 cm long were used in the experiments.

The elongation growth of segments 1 cm long was measured in a fluid medium in a flow chamber with the aid of a horizontal microscope [4, 1]. The measurements were made in one-minute intervals. The curves presented, showing segment growth progress, are each averages of at least 6–8 individual experiments. The curve representing average growth is depicted. Nitrogen-saturated solutions were prepared with "Stickstoff spezial" (Messer-Griesheim Co., Dortmund), which was cleaned of traces of oxygen with the aid of an Oxisorb cartridge (chromium trioxide on silica gel).

In order to determine endogenous NAD(P)H/NAD(P) level, coleoptile segments weighing 1 g were infiltrated with aqua bidest (removing air pockets inside the coleoptile cavity), and then gassed with N₂ or air in 60 ml aqueous bidest in a 30 °C water bath. After incubation the coleoptiles were gathered up with a sieve and immediately homogenized in a mortar with liquid nitrogen. When determining the amount of NAD(P)H the homogenate was transferred to an 11 ml solution of 50% alcoholic 0.1 M KOH at 60–80 °C and stirred 5 min. When determining the amount of NAD(P) 11 ml of 50% alcoholic 0.1 M HCl were used for each homogenate sample. The extracts were then quantitatively transferred into a centrifuge beaker (rinsing with 2 ml of the alcoholic solution). The extracts were cooled to 0 °C and centrifuged 10 min (39000×g); the supernatant volume was measured in a graduated cylinder; 8 ml thereof were added to 2.5 ml of a buffer solution (0.5 M triethanolamine-hydrochloride, 0.4 M KH₂PO₄, 0.1 M K₂HPO₄, 3 H₂O; ca. pH 5.8). The NAD(P)H sample was adjusted to a pH of 7.4 to 7.6, the NAD(P) sample to a pH of 7.2 to 7.4 and then filtered into 20 ml volumetric flasks. The NAD(P)H samples were titrated with a 25 ml of 9.2 mM DPIP solution, oxidizing any remaining reducing substances.

The NAD(H) and NADP(H) tests were carried out according to a modified method of Slater and Sawyer [5] and Wagner et al. [6]. The oxidized nucleotides were reduced in the presence of alcohol dehydrogenase (Boehringer Co., 200U/mg; 1:10 dilution) by adding alcohol; hydrogen is then transferred in nonenzymatic reactions to PMS (12.5 mg/ml; freshly prepared and kept in the dark) and from that substance to DPIP (2.5 mg/ml). Since the oxidized nucleotides are constantly being enzymatically reduced anew, the speed of DPIP reduction (at 610 nm) is a measure of nucleotide concentration.

NADH cuvette test sample composition: 0.8 ml aqua bidest., 1.5 ml phosphat-buffer (0.34 M KH₂PO₄ + K₂HPO₄ · 3 H₂O; pH 7.4), 0.05 ml ethanol, 0.05 ml neutralized 50% alcoholic 0.1 M KOH (NADH) resp. HCl (NAD), 0.1 ml DPIP, 0.1 ml PMS, 0.3 ml extract. Measurement at 610 nm until extinction decrease is only slight (Eₓ); reaction is started with 0.1 ml alcohol dehydrogenase (Boehringer Co., 1:10 dil.). Extinction measured again after 5 min (E₁). 

\[ \frac{E₁ - E₂}{E₂} = \Delta E \]

Determination of NADH or NADP concentrations on the basis of calibration curves.

NADPH test sample composition: 0.8 ml aqua bidest., 1.5 ml Tris (0.14 M, pH 8); 0.05 ml neutralized 50% alcoholic 0.1 M KOH; 0.05 ml glucose-6-phosphate (52 mg/ml); 0.1 ml DPIP (2.5 mg/ml); 0.1 ml PMS (12.5 mg/ml); 0.3 ml extract. Measurement at 610 nm after 20 min (E₁), then addition of 0.1 ml glucose-6-phosphat dehydrogenase (Boehringer; 1 mg/10 ml); E₂ after 5 min (E₂ - E₁ = ΔE). Determination of NADPH concentrations on the basis of calibration curves.

In order to determine the endogenous ATP concentration the coleoptile segments were first homogenized in a mortar in liquid N₂. The homogenate was transferred with a plastic scraper into ca. 20 ml boiling Tris buffer (20 mm, pH 7.55) and boiled for 3 min, subsequently filtered into an ice cold 20 ml volumetric flask and filled up with aqua bidest. Luciferin-luciferase (Lumit HS, ABIMED Co., D-4000 Düsseldorf 1) was used to measure ATP (measuring instrument: LKB, Luminometer 1215). The ATP content was calculated on the basis of a calibration curve.

**Determination of lactate, malate, pyruvate, citrate and aspartate**

1 g of coleoptile segments were treated as described above (determination of NADH) and, after
homogenization in liquid N₂, immediately transferred into 20 ml of boiling 80% ethanol to be boiled for 3 min. Subsequently the solutions were centrifuged (39000 x g) for 15 min at 0°C. The supernatant was filtered into a round flask, concentrated in vacuo and then filled to 5 ml with 50% ethanol. The acids were assayed enzymatically as described in refs. [7] and [8].

**Determination of cell sap pH**

Treated coleoptile segments (see above) were homogenized in liquid N₂, transferred to centrifuge beakers and centrifuged for 20 min at 0°C (39000 x g). The pH of the supernatant ("cell sap") was measured.

**Results**

**I. The ANA effect phenomenon**

In previous work [1] the ANA effect was described in 1 cm coleoptile segments immersed in a citrate-phosphate buffer solution. Figure 1 shows that the effect can proceed in aqua bidest as well. Certain exogenous ions and substrates are therefore not necessary for the effect to proceed; they can, however, affect the phenomenon quantitatively (Hager, unpublished). The anomalous increase of elongation growth is already quite demonstrable after 5 min of anoxia. This increase lasts longer for coleoptile segments in aqua bidest than for those in the buffer (Figs. 2 and 3).

**II. pH-dependence of the ANA effect**

Auxin induced elongation growth of coleoptile segments is increasingly repressed as the pH of the external solution rises [4, 9, 10]. The ANA effect, in agreement with this observation, is also depressible with increasing pH values of the external solution (Fig. 4). A drastic depression is already noticeable at pH 7.5. However, different buffer solutions are differently effective. Of buffers (5 mM) with a pH of 8.0, diethanolamine/HCl, Tris/HCl, and Na-Barbital/Na₂CO₃/HCl are stronger inhibitors than HEPES and BICINE buffers (data not shown).
Fig. 2. Growth rate of 1 cm coleoptile segments in aqua bidest. during and after a 20 min anaerobic period.

Fig. 3. Growth rate of 1 cm coleoptile segments in a citrate-phosphate buffer (5 mM), pH 5.5 during and after a 20 min anaerobic period. The increase in growth rate is as great as that in the unbuffered solution (Fig. 2), but of shorter duration.
Fig. 4. Coleoptile segments growth bursts following anaerobiosis (ANA effect) are increasingly inhibited by increasingly alkaline buffers.

The pH dependence of anoxia-induced growth is in accordance with that of auxin-induced growth; this suggests equal mechanisms.

**III. Respiratory poisons and uncouplers**

It is generally assumed that an active release of H⁺-ions into the cell wall compartment participates in the triggering of elongation growth [4, 11-13]. The participation of an H⁺-ATPase is probable. From Figs. 5 to 9 it becomes apparent that the usual array of inhibitor substances capable of attacking mitochondrial metabolism at different points such as KCN, azide, arsenate and amytal can depress the ANA effect. In the case of amytal, inhibition takes
place at higher concentrations (1 mM) because of the substances poor penetrance. Rotenon shows no inhibition of the ANA phenomenon. A weak supplementation of the ANA effect by low concentrations of the respiratory poisons KCN and Na-arsenate is perhaps due to the presence of monovalent cations in the added solutions which cause an increase in the ANA effect (Hager, unpublished).

These findings on the inhibitory action of respiratory poisons on the ANA effect do not rule out, however, that the energy for the growth inducing H⁺ release is provided by redox energy. NADH, for example, accumulated during anaerobiosis (see chapter V) could in this case set plasmalemmal electron transport (via flavines and cytochromes) in motion as soon as the terminal electron acceptor oxygen is on hand again. H⁺ could be released to the outside in the process. Since such an electron transport system could also be depressed with the inhibitors mentioned above, a choice between the participation of a redox pump or an H⁺-ATPase during elongation growth and specifically in the base of the ANA effect, cannot be made on the basis of their effects alone.

The extraordinarily strong repression of the growth "burst" with the uncoupling, protonophoric substance CCCP (Fig. 10) can be ascribed to the inhibition of ATP synthesis in the mitochondria just as it can to the removal of the proton gradient across the plasmalemma responsible for growth.

IV. Change in ATP concentration

As shown in Fig. 11, the initial concentration of ATP in young coleoptile segments is relatively high (130 nmol/g fresh weight). In a nitrogen atmosphere this value decreases in a few minutes to about 30 nmol/g, but just one minute after reintroduction of O₂ 125 nmol ATP/g fresh weight is measured. This means that the values of control plants exposed to air have almost been attained in this short time. Thus, in the growth "burst" phase, the level of ATP is not higher. It can be shown, however, (Hager and Abele, unpubl.) that CO₂ release i.e. respiration rate and thus probably ATP turnover as well, are higher at the beginning of the growth "burst".

V. Change of NADH/NADPH ratio

The NADH content during the ANA effect shows an opposite tendency to the measured changes in ATP concentration (Fig. 12). Coleoptile segments in aerobic conditions contain around 10 nmol NADH per gram of fresh weight. The NADH content value climbs to nearly twice this value in anaerobic conditions. After reintroduction of air, however, it takes more than 20 min to return to the original value — in contrast to the findings in the case of ATP. If one assumes that cellular NADH turnover has a half life time similar to that of ATP of 1—2 min, the twenty minute long surplus of NADH seems to indicate a low readiness of mitochondria to take up and utilize

![Fig. 6. Inhibition of the ANA effect in coleoptile segments with KCN.](image-url)

![Fig. 7. Influence of different concentrations of sodium arsenate on the ANA effect in coleoptile segments.](image-url)
this NADH. The alternative would be that new NADH is constantly being provided during the growth burst. This could be effected with lactate accumulated during anaerobiosis (Lactate + NAD → Pyruvate + NADH), see Chap. VI.

In contrast to the NADH level the NADPH concentration drops from 15 to 2 nmol/g fresh weight within 15 min under conditions of anoxia; following the resupply of air it takes 15 to 20 min till the value of the air control has been attained again.

VI. Change in pH and lactate concentration

During oxygen deprivation an accelerating drop in cell sap pH of the coleoptiles is observable
Fig. 10. Inhibition of the ANA effect by the uncoupler CCCP.

Fig. 11. ATP concentration in coleoptile segments under anaerobic and aerobic conditions.

(Fig. 13). This striking change is explained by the increase in lactate concentration shown in Fig. 14. Both processes can be reversed by reintroduction of O₂. However, even after 40 min, the lactate concentration is not reduced to that measured in air controls. Vacuole storage of lactate is a plausible explanation. In contrast to lactate, other acids such as citrate, malate and aspartate show no significant changes in concentration in response to aerobic or anaerobic conditions (Fig. 14).

Discussion

A short period of anaerobiosis has an effect on coleoptile segments similar to the addition of auxin (IAA) in optimal concentration. The induced elongation growth shows the same strict dependence on pH of the external solution in both cases: the more alkaline the solutions, the stronger the growth will be depressed (Fig. 4). This is an indication that the
ANA effect requires an increase in proton concentration in the cell wall compartment to trigger elongation growth, as is presumed for auxin-induced elongation growth [4, 11, 14–16; reviews: 12, 13].

As an immediate cause of this post-anaerobic growth effect, chemical changes in the cells during anaerobiosis come into consideration. Several marked changes were demonstrated.

1. The drastic lowering of the ATP level during anaerobiosis could lead to a sort of Pasteur effect, that is, to an activation of glycolytic enzymes like phosphofructokinase, pyruvate kinase or pyruvate dehydrogenase [17]. After resupplying O$_2$ glycolytic products would flow into the citric acid cycle in greater quantity. An enhanced energy availability for growth processes could be the result. However, such an effect could probably not last 20–30 min, because its controlling ATP or P$_i$ level is adjusted back to the normal niveau a few minutes after oxygen supply (Fig. 11).

2. The NADH level rises during anaerobiosis (even though one would assume that increased NADH concentration would immediately be absorbed by the formation of lactic acid). The accumulated NADH is returned to its original value upon resupply of oxygen; surprisingly, this process is quite slow: it takes almost 20 min for the original concentration to be reached, which is as long as the hyperelongation growth lasts.

This probably means that cytoplasmic NADH either cannot be transported or only with great difficulty into the mitochondria, when the mitochondria are capable of producing NADH themselves in the Krebs cycle in the presence of oxygen. Furthermore, it can mean that NADH is supplied with the aid of lactate dehydrogenase in the conversion of lactate to pyruvate during the post-anaerobic growth phase.

The question is whether NADH is a primary cause in the triggering of the growth burst. It is plausible that the H$^+$ export necessary for elongation growth is effected with a redox pump (not an H$^+$-ATPase). Since there are indications of the presence of cytochromes or flavines in the plasmalemma and the ER [18–20], an electron transport from NADH along these molecules to a terminal oxidase (in the presence of O$_2$) coupled with a H$^+$ export is thinkable [21]. In this case the ANA effect should not be repressible with inhibitors of oxidative phosphorylation; however, this is not the case. The possibility that the inhibitors used also depress
the postulated plasmalemma or ER electron transport cannot be ruled out.

3. It is striking that the NADPH concentration falls in almost the same amount as the NADH concentration rises. This could mean that a transhydrogenase is at work (review: [22]) which can also work reversibly (NADH + NADP+ ⇌ NAD+ + NADPH). Such an enzyme has been demonstrated in spinach chloroplasts, for example [23]. It is known of the transhydrogenase in Pseudomonas that it is controlled by pH and calcium concentration [24]. The accumulation of NADH at the expense of NADPH could also mean that a phosphatase is carrying out the conversion of NADPH to NADH (NADPH → NADH + P). This would be the reverse of the NAD-kinase reaction, a Ca2+ controlled reaction [22] which can also work provided in greater quantities could trigger increased oxidation of carbohydrates could take place following anaerobiosis. The ATP thus provided in greater quantities could trigger increased growth mediated by H+-ATPases.

4. In contrast to the levels of other organic acids, that of lactic acid rises drastically during anaerobic treatment (Fig. 14). Thus cells switch to glycolysis immediately. As a result of this acid accumulation a considerable drop in the cytoplasmic pH can be observed (such effects have also been noted in other higher plants during anaerobiosis; see [28], for example). This drop in pH (Fig. 13) could favor the active secretion of H+ ions during the aerobic period thermodynamically; however, it is more probable that it causes changes in enzyme activities [29]. In this context, the ATPases of microsomal membranes, which seem to be responsible for H+ secretion [11] show a striking pH-dependence. It has been shown that ATPases of the plasmalemma and ER have a pH optimum of 5 to 7 [30–38]. If one assumes that the cytoplasmic pH is usually around 7, every lowering of pH in the cytoplasm would have to lead to drastic increases in H+-ATPase activities. Increased H+ secretion and increased growth would follow. If this is the responsible mechanism, all possibilities of lowering cytoplasmic pH would have to lead to an oxygen-dependent stimulation of growth. Experiments (unpublished) carried out up to now argue for such a mechanism.

5. Finally the interpretation of so-called emergent growth, found by [2, 3] in pea segments following anaerobiosis, can be discussed. These authors trace it from a release of auxins from a cell compartment or a bound form during anaerobiosis. They conclude this mainly because of greater elution rates of added [2-C14]IAA under anaerobic conditions. This fact can possibly be also explained in that IAA externally administered to segments is to a great extent decarboxylated enzymatically to 3-methyleneoxindole in the presence of O2 [39–42], especially on wound surfaces, and thus cannot be given off again (3-methyleneoxindole remains bound on or in the cells). A further argument against the induction of emergent growth by newly provided auxins is that this growth begins without the usual lag phase.

Summing up, one can say that the intracellular changes mentioned in points 2, 3 and especially 4 are probable causes of the ANA effect.

The following can be said about the range of plants exhibiting the ANA effect. Not only the coleoptiles but also Helianthus hypocotyls have similar but shorter growth “bursts” (Hager, unpubl.). Pea stem emergent growth [2, 3], mentioned above, lasts hours on the other hand.

An increased elongation growth of light-germinated rice seedlings must probably be mentioned in this context [43, 44]; these seedlings begin to etiolate under anaerobic conditions in spite of the presence of light.

An etiolation can also be caused by suppressing respiration of light germinated rice seedlings with amytal [45].

The growth effects observed by T. Fujii et al. [46, 47] in Avena coleoptiles seem to have causes similar to the ANA effect. The authors find an increase in elongation growth when oxygen supply is reduced. The growth rate is greater at a oxygen concentration of 6–8% than at one of 20% O2. In all of these cases the weakening of respiration and an accumulation of fermentation by- and end-products could lead to increased elongation growth as they do in the ANA effect.

The acceleration of growth in conditions of low O2 partial pressure seems plausible for a plant, from an ecologic point of view, in order to get to better living conditions.
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