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Loss of NAD(P)-Reducing Power and Glutathione Disulfide Excretion at the Start of Induction of Aerial Growth in *Neurospora crassa*

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When exponentially growing hyphae of *Neurospora crassa* in aerated liquid cultures are filtered and the resulting mycelial mat is exposed to air, aerial hyphae develop and synchronous conidiation is obtained. The hyphae in direct contact with air adhere to each other within minutes and form aerial hyphae during the following 12 h; the hyphae which are not in direct contact with air do not adhere to each other and do not form aerial hyphae. Previous data indicated that oxidative stress was generated in the adhering hyphae; proteins and specific enzymes were found to be oxidatively modified and degraded. In this work, we report a dramatic fall in the reduced-to-oxidized ratio of NAD and NADP coenzymes during the first 6 min of exposure to air. This drop did not occur in a mycelial mat exposed to a N_2-enriched atmosphere. Adding a carbon source to the mycelial mat did not abolish the loss of NAD(P)-reducing power. After the initial fall, the reducing levels of the coenzymes returned to the starting value in about 30 min. A peak of extracellular glutathione disulfide occurred simultaneously with the loss of NAD(P)-reducing power. The reducing power loss and the excretion of glutathione disulfide are thought to be consequences of a hyperoxidant state; the adhesion of hyphae is thought to be a response to the hyperoxidant state.

When aerated liquid cultures of *Neurospora crassa* are filtered and the resulting mycelial mat is exposed directly to air, aerial hyphae develop and produce conidia in a synchronous manner (23, 24). The hyphae in direct contact with air adhere to each other, forming a tight tissue that can be easily separated from the aerial hyphae and the loose mycelia below (5, 24). The adhering hyphae of the upper layer (UL) of the mycelial mat form the aerial hyphae, while the loose hyphae of the lower layer (LL) contribute to aerial growth only in an indirect manner (24).

In the UL, protein catabolism is intensified at the initiation of the developmental process (6). As indicated by the carbonyl content of purified protein (3, 15, 17, 22), proteins are oxidized as soon as the mycelium is exposed to air (25). Glutamine synthetase of the UL, but not of the LL, was found to be oxidatively modified when analyzed 30 min after the mycelial mat was exposed to air (2a). This modification of the enzyme was similar in every aspect to that observed in an aerated liquid culture without a carbon source, or when the purified enzyme was modified in vitro by oxygen radicals (1). The in vitro-oxidized glutamine synthetase was shown to be more labile to the proteolytic activities of the endogenous cell extract than the unmodified enzyme (1).

These results suggested to us that in the UL a hyperoxidant state is generated as soon as the mycelial mat is exposed to air. A hyperoxidant state can be defined as an unstable physiological state in which the reactive oxygen species generated surpass the capacity of the cell to neutralize them (9). The reactive oxygen species react with the cellular components, causing protein oxidation (1, 2, 8, 19, 21, 28), mutation and cleavage of nucleic acid (11), and lipid peroxidation (14). Two expected consequences of oxidative stress are analyzed in this study, loss of reducing power (13) and excretion of glutathione disulfide (20).

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Extraction and measurement of coenzymes. Mycelial mats were made with 12-h cultures of the wild-type 74A strain raised at 30°C from an inoculum of 10^6 conidia per ml of Vogel's medium (26) supplemented with 1.5% sucrose. For the extraction of coenzymes, three mycelial mats, each obtained from 25 ml of culture, were processed together for either oxidized or reduced coenzyme extraction. Three-layer mycelial mats were used to obtain the LL. Each layer was made by filtering 125 ml of liquid culture and was separated from the other layers by filter paper. After different lengths of time of exposure to air, the UL of a three-layer mycelial mat was discarded and the remaining two LL were used for the determination of oxidized and reduced coenzymes.

After exposure to air for various periods of time, the mycelial mats were homogenized at 4°C with 20 strokes of a Potter-Elvehjem homogenizer in 1.5 ml (2.5 ml for the LL) of either 0.6 M perchloric acid for the oxidized coenzymes or 0.25 M NaOH for the reduced coenzymes, by the method of Jacobson and Jacobson (12). The acid and alkaline cell extracts were filtered through Millipore HA filters (pore size, 0.22 μm), and immediately after extraction, the quantities of the coenzymes were determined by high-pressure liquid chromatography (HPLC). Protein levels were determined by the method of Lowry et al. (16).

The HPLC method of Hartwick et al. (10) was adapted to our Waters Associates HPLC facilities. Samples, usually 50 μl each, were separated in a Z-Module compressed μBondapack C18 cartridge by passage through a linear 0 to 60% gradient formed by adding an increasing volume of a methanol-water (3:2 [vol/vol]) solution to a 0.02 M solution of

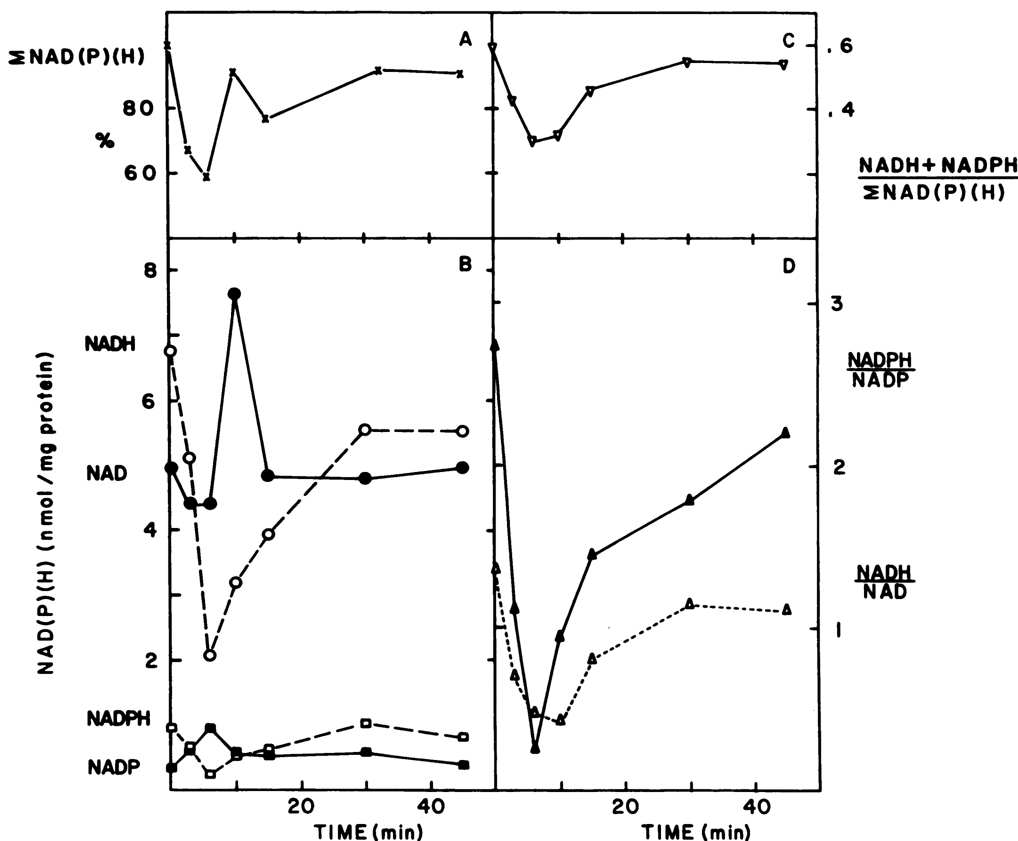

FIG. 1. NAD(P)(H) coenzymes of the UL during the first minutes of exposure to air. (A) Sum of the NAD(P)(H) coenzyme concentration expressed as a percentage of the initial total NAD(P)(H) concentration, which corresponds to the concentration in a mycelium at the end of the exponential growth phase. (B) Coenzyme concentrations of NADH, NAD, NADPH, and NADP. Mean values of 3 to 11 (usually 5) determinations from three experiments are shown. The standard deviation was less than 25% of the mean values; the mean standard deviation was 12% of the mean values. (C) Reductive NAD(P)(H) charge, calculated as the sum of the reduced coenzyme concentrations divided by the sum of the reduced plus oxidized coenzyme concentrations. (D) Ratios of the reduced to oxidized coenzyme concentrations, NADH/NAD and NADPH/NADP.

KH_2PO_4, pH 5.6. The gradient was made with an automated gradient controller and run at a constant flux of 3 ml/min for 38 min. A_{254} and A_{340} were analyzed with a Data Module microprocessor. To calculate the concentrations of the coenzymes, standard curves were made, using purified coenzymes from Sigma. The relationships were linear for A_{254} of 0.1 to 1 nmol of NAD or NADP and for A_{340} of 0.15 to 2 nmol of NADH or NADPH. One nanomole of NAD or NADP gave a signal of 1,300 and 1,720 V, respectively, in the Data Module microprocessor. One nanomole of NAD or NADP gave a signal of 435 and 395 V, respectively.

Oxidative state of the NAD and NADP coenzymes in the UL. Mycelial mats were exposed to air for various times and the level of oxidized or reduced NAD and NADP coenzymes was determined by HPLC. The initial values, time zero in Fig. 1, 2, and 4 to 6, correspond to the coenzyme concentrations found in the hyphae at the end of the exponential growth phase. In these cultures, the concentration of NAD coenzymes was about nine times the concentration of NADP coenzymes. The percentages of reduced dinucleotides in the growing hyphae were 57 for NAD and 73 for NADP. The total NAD(P)-reductive charge, e.g., the ratio of the sum of coenzymes in a reduced state to the total NAD(P)(H) coenzyme content, was close to 0.6.

The concentration of NADH and NADPH coenzymes diminished sharply during the first 6 min of exposure to air. During the following 30 to 60 min, reduced coenzymes recovered and almost attained the initial values (Fig. 1B). The oxidized coenzyme concentrations exhibited the opposite: they increased sharply during the first 6 to 10 min of air exposure and then progressively returned to the initial values in the following 20 to 30 min (Fig. 1B). The loss of NADH together with a reproducible lag in the rise of NAD affected the total coenzyme content, which decreased about 40% after 6 min of incubation (Fig. 1A). The reduced coenzyme/oxidized coenzyme ratios showed a dramatic fall during the first minutes of exposure to air (Fig. 1D). These low ratios were reflected in a decrease in total NAD(P)-reductive charge to half the initial value (Fig. 1C).

After the first 6 to 10 min of exposure to air, a relatively slow recovery of reduced coenzyme/oxidized coenzyme ratios was observed, reaching values similar to the zero time values (Fig. 1B). Recovery was also observed in the total reductive charge (Fig. 1C) and the total coenzyme content (Fig. 1A).

Oxidative state of the NAD(P) coenzyme in the mycelial mats at low-oxygen atmospheres. Hypha adhesion starts as soon as the hyphae are directly exposed to air, with the whole process requiring less than 30 min (24). When mycelial mats were exposed for 12 h to an atmosphere with a constant

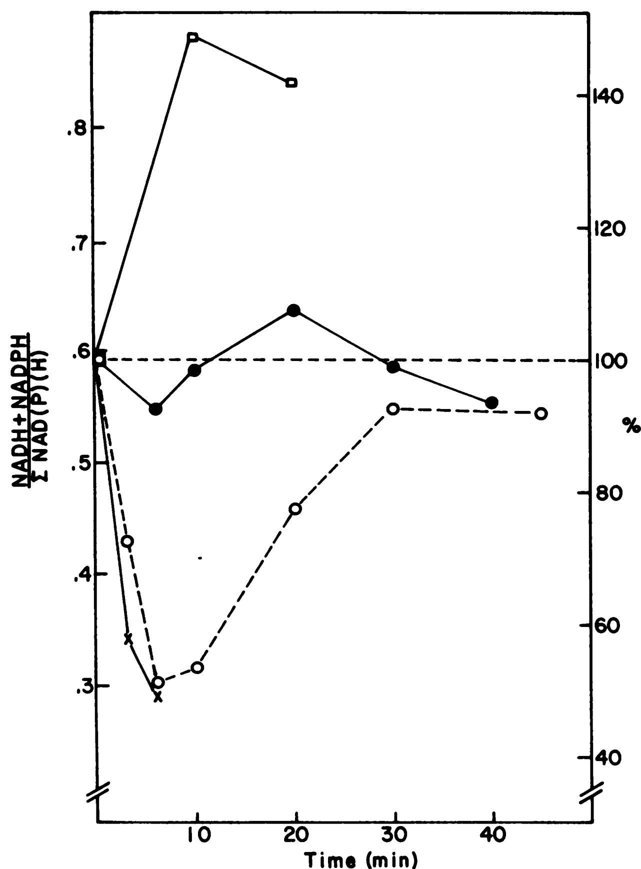

FIG. 2. Reductive charges of mycelial mats under different oxygen conditions. Symbols: □, N_2-enriched atmosphere; ×, O_2-enriched atmosphere; ○, UL; ●, LL.

flux of N_2, adhesion of the hyphae did not occur and growth of aerial hyphae did not take place. When these mycelial mats were exposed to air for another 12 h, adhesion of the hyphae and formation of aerial hyphae and conidia were observed.

The total NAD(P)-reductive charge in mycelial mats exposed for 10 or 20 min to a N_2-enriched atmosphere increased considerably (Fig. 2). This was due to a marked rise in the reduced-to-oxidized ratio of both coenzymes. These high ratios were due to a moderate increase in the reduced coenzyme concentration and a decrease of about four times in the oxidized coenzyme concentrations. Total NAD(P)(H) content diminished nearly 30%. When a flux of O_2 instead of N_2 was passed over the mycelial mats, the total NAD(P)-reductive charge declined rapidly (Fig. 2). This was due to a loss of the reduced coenzymes and an increase in the oxidized coenzymes. The reduced-to-oxidized ratio of NAD(P) coenzymes was even lower than in the mycelial mats exposed to air.

Because the LL is relatively isolated from air by the UL, it is probably in a less aerobic condition than the UL. To confirm this, O_2 permeation through mycelial mats of different thickness was measured with an oxygen meter and a Clark electrode. Mycelial mats of different thickness were made directly on the electrode tip, and O_2 concentration was recorded for 30 min. It was observed that O_2 permeation diminished very rapidly during the first minute and then more slowly until a plateau was reached at about 5 min.

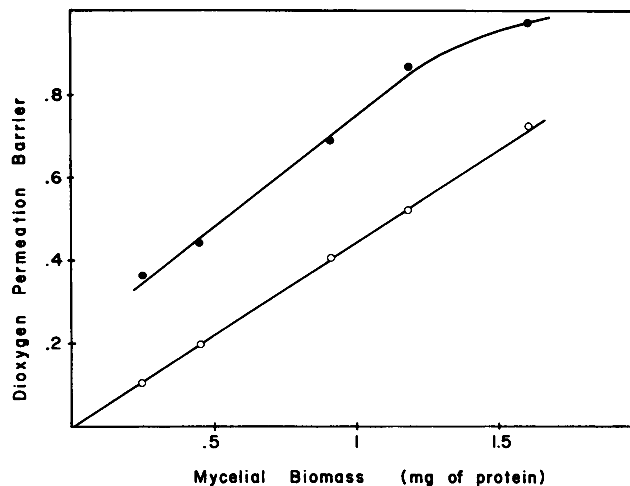

FIG. 3. Dioxygen permeation barrier related to the thickness of the mycelial mat. The differences in O_2 concentration measured at 0.5 (○) or 5 (●) min (plateau value) are shown. The concentration of O_2 in air-saturated water at room temperature was set at zero. The vertical scale shows 1 minus the difference in O_2 concentration.

Permeation of O_2 at 0.5 and 5 min (plateau level) decreased with increasing thickness of the mycelial mat (Fig. 3).

The LL represents a more physiological condition than the exposure of the hyphae to a N_2-enriched atmosphere and is an appropriate control for the changes in the oxidative state and coenzyme concentration that take place in the UL. The reduced coenzyme/oxidized coenzyme ratios, particularly NADPH/NADP, rose between 6 and 20 min of incubation (Fig. 4D). Similar to the N_2 condition, the increase in this ratio was due to both a loss in the oxidized coenzymes and a gain in the reduced coenzyme concentration (Fig. 4B). After an initial loss of 30% (see below), the total coenzyme content increased steadily but never reached the initial value (Fig. 4A). The rise in the reduced-to-oxidized ratio of both coenzymes augmented the total reductive charge which was higher than the initial value at 20 min of incubation but decreased toward the end of the incubation period (Fig. 4C).

An initial drop in the NADH/NAD ratio was observed at 6 min of incubation, but it was about one-third of that detected in the UL (compare Fig. 1D and 4D). This drop was reflected in the total reductive charge (Fig. 4C) and particularly in the total coenzyme content. The initial drop in the NADH/NAD ratio of the LL was probably due to filtration of the mycelium, which momentarily increased aeration of the hyphae.

Oxidative state of the NAD(P) coenzymes in a UL with added carbon source. The foregoing experiments indicate that the marked loss of the reduced coenzyme/oxidized coenzyme ratios was related to the exposure of the mycelium to air. Since the concentration of reduced coenzyme depends on both the aeration of the mycelium and the availability of carbon sources, the coenzymes were measured in the UL in the presence of added sucrose. Sucrose at a concentration of 1% or more stimulated aerial hypha formation; at concentrations below 1%, it had the opposite effect (24). The NAD(P)(H) concentrations were determined in the UL with 0.15 or 1.5% sucrose added. As shown in Fig. 5 and 6, in the presence of sucrose, regardless of the concentration included, there was a marked loss in the

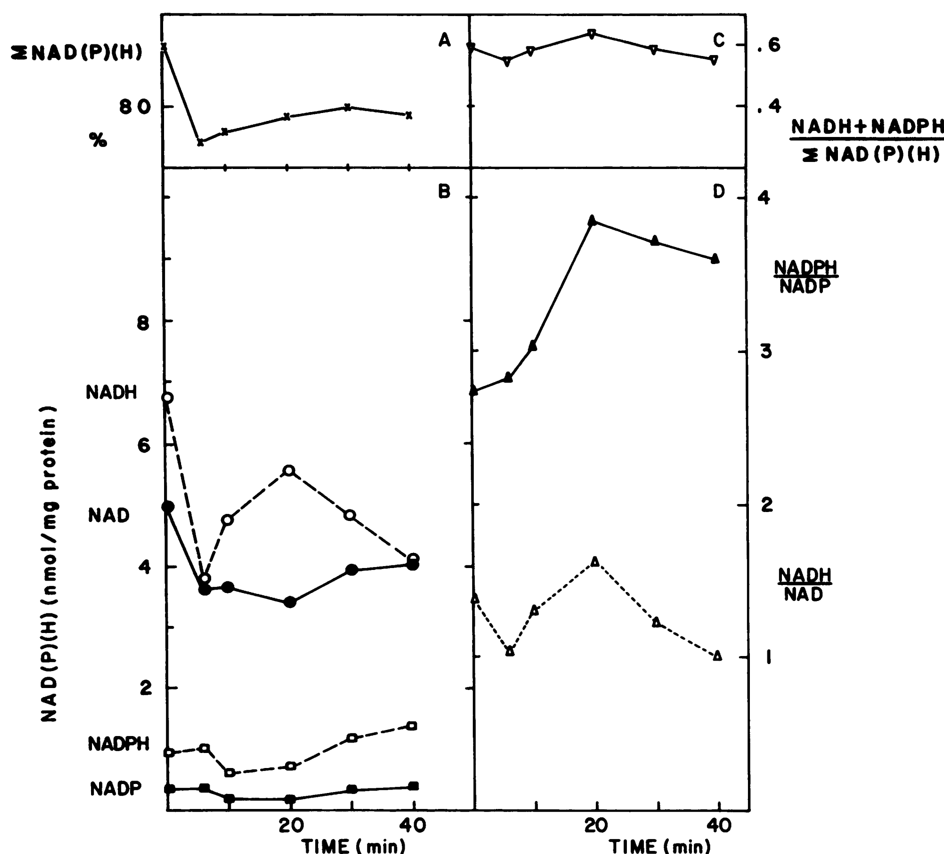

FIG. 4. NAD(P)(H) coenzymes of the LL. (A) Percentages of the initial total content. (B) Coenzyme concentrations. The mean values of three to six (usually four) determinations from three experiments are shown. The standard deviation was less than 20% of the mean value; the mean standard deviation was 7.9% of the mean values. (C) Reductive NAD(P)(H) charge. (D) Ratios of the reduced to oxidized coenzyme concentrations.

concentration of reduced coenzymes, particularly of NADH, during the first 6 min of exposure to air (Fig. 5B and 6B). This loss is reflected in the total amount of coenzymes (Fig. 5A and 6A). With 0.15% sucrose, the oxidized coenzymes increased considerably, giving relatively low NADH/NAD ratios and rather low NADPH/NADP ratios (Fig. 5D). With a 1.5% sucrose concentration, there was a marked transient increase in the NADH concentration, giving a high NADH/NAD ratio after 10 min of exposure to air (Fig. 6D), which influenced the total NAD(P)-reductive charge (Fig. 6C). The NADH concentration then decreased, as did the total reductive charge and the NADH/NAD ratio, giving values that were lower than the initial values (Fig. 6B, C, and D, respectively).

Thus, the marked decrease in the total NAD(P)-reductive charge observed in the UL is not explained by a lack of carbon skeletons. A transient decrease in carbon skeletons due to a reduced transport of the carbon source is unlikely because permeases are active in the UL since labeled glucose or amino acids are incorporated readily into the hyphae (24). Also, stored glycogen in the hyphae is rapidly consumed in the UL when no external carbon sources are added (6a).

A high influx of O_2 in the hyphae exposed to air could be thought to stimulate the respiratory chain and to consequently increase consumption of reductive charge and oxidation of carbon skeletons. Such a condition could result in a transient lack of available carbon skeletons and depletion of reduced coenzymes. However, these events cannot account for the rapid loss of NAD(P)-reducing power, because the total respiratory activity of mitochondria was found to be constant or to decline under hyperbaric O_2 conditions (7). In fact, respiration does not increase because the cytochrome oxidases from most sources are essentially saturated even at the lowest detectable concentration of dissolved O_2. The reported K_ms for dioxygen of cytochrome oxidase from different sources range between 10^{-6} and 10^{-8} (18).

Glutathione excretion. To further substantiate that the dramatic changes in the NAD(P)-reducing power reflected an unbalanced redox state in the hyphae exposed to air, glutathione disulfide excretion in the UL was measured. Many cells respond to oxidizing conditions by excreting glutathione disulfide (20). After different lengths of time of exposure to air, 2 ml of N_2-saturated 100 mM phosphate buffer, pH 7.4, was added to each mycelial mat and shaken for 5 s while a flux of N_2 was passed over. The buffer was recovered and filtered through Whatman filter paper to get rid of remaining hyphae. This wash was repeated a second time. The glutathione content was then determined by the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of glutathione reductase (0.5 U/ml) and NADPH (0.24 mM) (20). To determine the proportion of glutathione disulfide, samples in some experiments were first treated with *N*-ethylmaleimide, passed through a Sep-

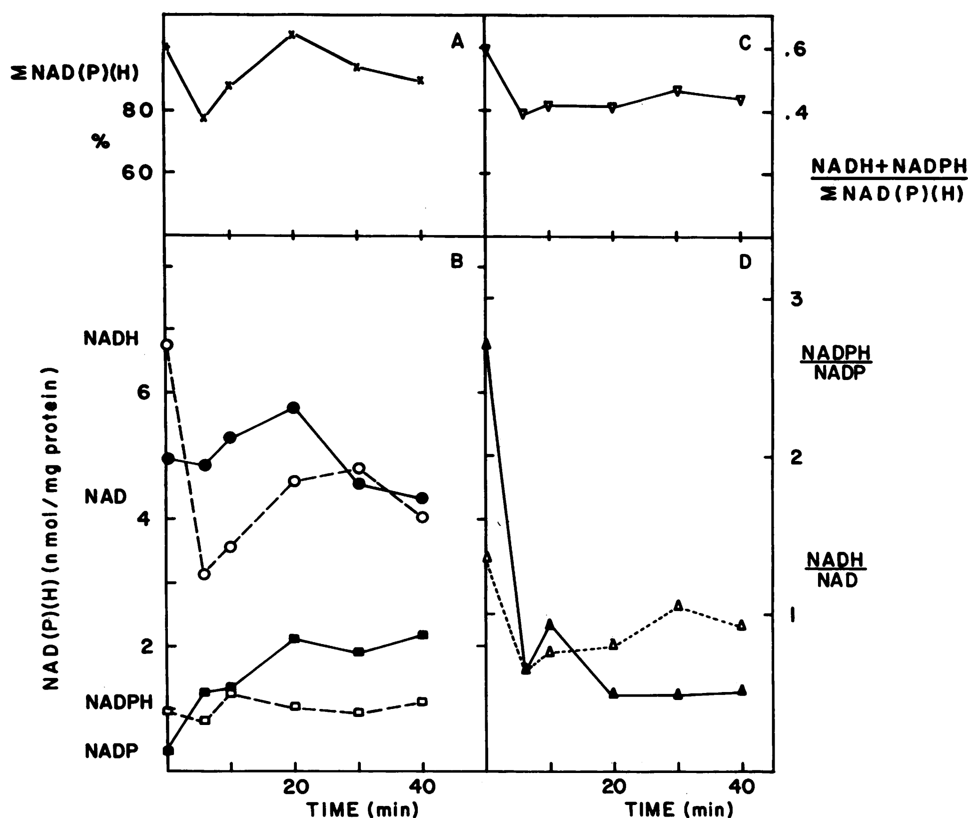

FIG. 5. NAD(P)(H) coenzymes of the UL in the presence of 0.15% sucrose. (A) Percentages of the initial total content. (B) Coenzyme concentrations. The mean values of 3 to 17 (usually 6) determinations from three experiments are shown. The standard deviation was less than 23% of the mean value; the mean standard deviation was 13.5% of the mean values. (C) Reductive NAD(P)(H) charge. (D) Ratios of the reduced to oxidized coenzyme concentrations.

pack, and then the concentration of gluthatione was measured.

The hyphae of *N. crassa* exposed to air excreted glutathione very rapidly. Extracellular glutathione peaked during the first 10 min after exposure to air and then decreased during the following 30 min (Fig. 7A). The extracellular glutathione was 70 to 97% glutathione disulfide. This peak of extracellular glutathione was not observed when the mycelial mat was exposed to a N_2-enriched atmosphere (Fig. 7A). When the mycelial mats were washed with phosphate buffer containing either 0.15 or 1.5% glucose (or sucrose), the peak of glutathione excretion persisted but was somewhat less pronounced than that without carbon source (Fig. 7).

Glutathione excretion was related to the presence of O_2 and was particularly high when a mat was washed several times in the presence of oxygen (data not shown). Washing the mycelial mats several times under a N_2-enriched atmosphere did not increase glutathione excretion. Thus, the wash itself elicited glutathione excretion only in the presence of O_2.

Extracellular glutathione could be due to leakage caused by breakage of hyphae during the filtration procedure. Although leakage cannot be excluded, because the values at time zero were always higher than those for filtered growth medium, it does not explain the increase in extracellular glutathione, which was observed only when the mycelial mat was exposed to air.

Conclusions and interpretation of the data. A loss in NAD(P)-reductive charge was particularly marked in the UL compared with the LL. The difference between the UL and LL is that the former is in direct contact with air, while the latter is isolated from air by the UL. In the absence of air (e.g., a mycelial mat in a N_2-enriched atmosphere), loss in the reducing power was not observed. As expected, under this condition the concentration of reduced coenzymes increased, since anoxic states are known to increase the reduced levels of the NAD(P) coenzymes (7, 27). The O_2 determinations in mycelial mats of different thickness and the increase in the NAD(P)-reductive charge observed in the LL after 10 min of incubation indicates a microaerobic state in this layer. This layer does not form aerial hyphae (24); mycelial mats also did not form aerial hyphae under a N_2 atmosphere. The marked decrease in the total NAD(P)-reductive charge observed in the UL was not abolished by adding a carbon source, although it was less severe with 1.5% sucrose. There was only a transitory effect of the carbon source on the NADH/NAD ratios after 10 min of exposure to air, and the total NAD(P)-reductive charge remained low after 20 min of exposure to air.

The NAD(P) coenzymes have been measured in the conidium-producing and the non-conidium-producing areas of a mutant strain which exhibits rhythmic circadian conidiation when grown in agar plates. Although the experimental conditions used were different, the overall results were similar to ours, namely, the NAD(P)-reductive charge and

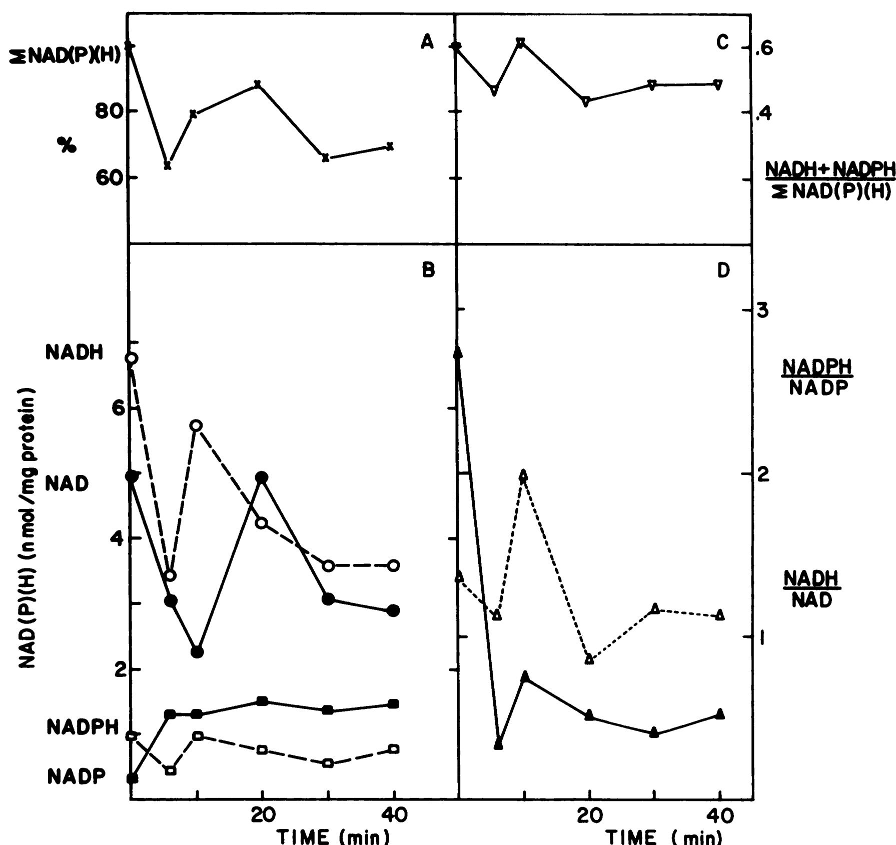

FIG. 6. NAD(P)(H) coenzymes of the UL in the presence of 1.5% sucrose. (A) Percentages of the initial total content. (B) Coenzyme concentrations. The mean values of 3 to 11 (usually 5) determinations from two experiments are shown. The standard deviation was less than 19% of the mean value; the mean standard deviation was 11.4% of the mean values. (C) Reductive NAD(P)(H) charge. (D) Ratios of the reduced to oxidized coenzyme concentrations.

the total NAD(P)(H) coenzyme content are lower in the conidium-producing areas than in the non-conidium-producing (growing) areas (4). Glutathione disulfide excretion has not been detected before.

The data presented are indicative of a rapid redox imbalance, which is compensated while the hyphae adhere to each other. These data and our previous results on the oxidation of enzymes and proteins (1, 2, 5) are consistent with the hypothesis that a hyperoxidant state is generated in the hyphae exposed to air (9). We think that this hyperoxidant state triggers the adhesion of the hyphae. Adhesion of the hyphae would cause a rapid reduction of O_2 permeation into the hypha aggregates as a result of a decrease in the volume-to-surface ratio, allowing cells to recover from the hyperoxidant state. The aggregate state is considered to be a differentiated state, which is stable until a second hyperoxidant state is generated that will lead to further differentiation of the hyphae. A recurrent peak of protein oxidation has been detected which occurred simultaneously with the appearance of the different cellular structures that lead to the formation of conidia (25). The possible relationship of hyperoxidant states to cell differentiation offers a very attractive hypothesis that could be useful for understanding many features of microbial cell differentiation. Thus, a general theory of microbial cell differentiation has been proposed (9).

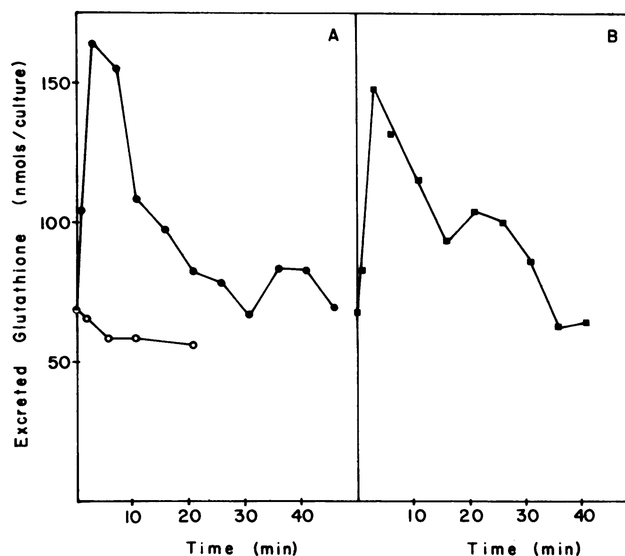

FIG. 7. Excretion of glutathione during the first minutes of exposure to air. (A) Mycelial mats washed with phosphate buffer at the start of incubation. Symbols: ●, exposed to air; ○, exposed to a N_2-enriched atmosphere. (B) Mycelial mats washed at the start with phosphate buffer containing 1.5% glucose (■). The mean values from two experiments are shown.

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