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# The Effect of Oxygen on NAD Breakdown in *Salmonella typhimurium*

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**The breakdown rate of NAD in *Salmonella typhimurium* was investigated both in aerobic and anaerobic conditions. After NAD is broken down to nicotinamide ring containing moiety, almost all the nicotinamide ring containing moiety recycles back to NAD pool. However almost none of the adenine containing moiety recycles back. We pulse-label the endogeneous NAD with [$^{14}$C]-adenine and [$^{3}$H]-niacin. The remaining [$^{14}$C]-radioactivity in NAD pool at each time was regarded as unbroken portion of NAD, where as that of [$^{3}$H] was served as a total amount of NAD to start with. Under aerobic condition, the half-life of NAD was around 2 hours. However, the breakdown rate was significantly reduced (around 3~4 fold) under anaerobic condition. The observation that under aerobic conditions, NAD turnover is considerably faster than under anaerobic conditions suggests that oxygen has important effect in NAD breakdown.**

**Key words: NAD breakdown, oxygen effect, anaerobic**

Molecular oxygen has an enormous effect on the metabolism of facultative bacteria. During growth aerobically, bacteria can obtain the maximum energy from a given carbon source by carrying out oxidative phosphorylation, using oxygen as the terminal electron acceptor. In the absence of oxygen, the cell utilizes mixed acid fermentation which leads to the formation of such end products as lactate, ethanol and fumarate. Therefore, the presence of oxygen leads to metabolic processes grossly different in terms of carbon utilization and electron flow from those which occur under anaerobic conditions. Although aerobic life style offers advantages (greater metabolic efficiency and faster growth rate), it is fraught with danger of oxidative damages: superoxide radicals and hydrogen peroxide, for example, may be released during the reduction of molecular oxygen (8). Such destructive compounds are detoxified by enzymes which are induced during aerobic growth. Superoxide dismutase (4) and catalase (6) inactivate superoxide radicals and hydrogen peroxide. A variety of other enzymes may be induced during aerobic growth to counteract the deteriorative effects of oxygen.

Toxic compounds may, to some extent, escape inactivation by scavenging enzymes, resulting in damage to cellular components. Enzyme systems to repair such damages might be in place. Clearly, then, molecular oxygen may effect a variety of metabolic pathways especially the carbon utilization and the electron flow as well as various repair systems (1).

NAD has a central metabolic role as an oxidation reduction cofactor. The metabolism of this important and versatile cofactor is quite complex (Fig. 1). The major branches of NAD metabolism in procaryotes include *de novo* synthesis of the cofactor, utilization of exogeneous precursors, and a cycle of NAD breakdown and resynthesis. NAD is known to be broken down into nicotinamide ring containing moiety and adenine ring containing moiety (7). What causes such breakdown of NAD molecule has not been answered yet.

One experimental approach to this problem can be to define growth conditions under which the rate of turnover is changed. This paper describes the effects of oxygen on the breakdown of NAD in *S. typhimurium*. Unexpectedly, the rate of breakdown in anaerobic conditions is quite different from that in aerobic conditions. The estimation of NAD breakdown rate which occurs under each growth condition is presented.

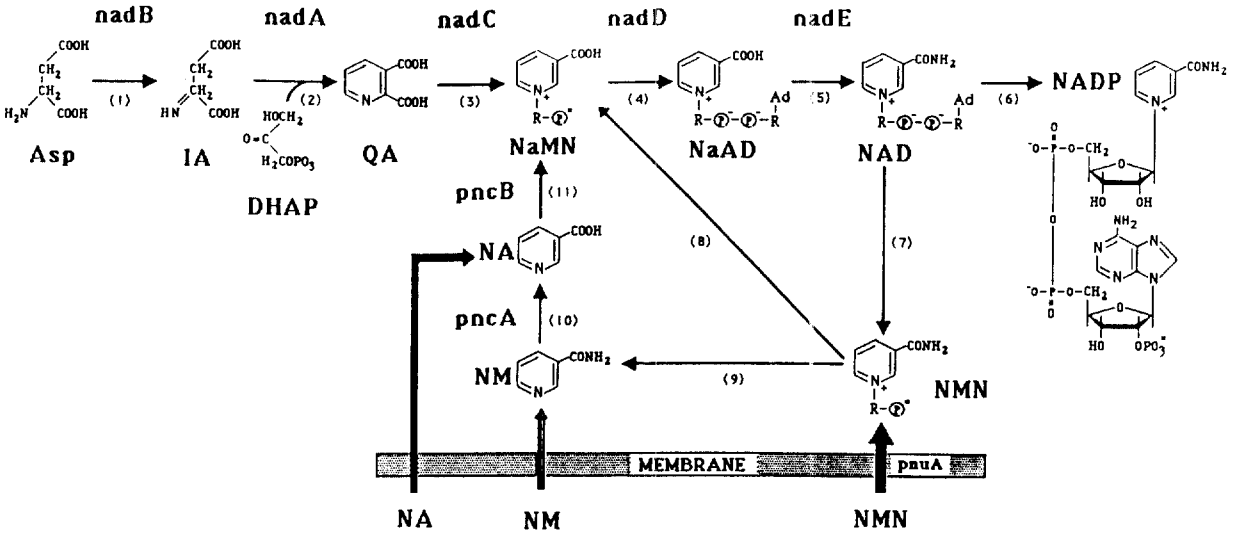


**Fig. 1.** The NAD metabolic pathway of *Salmonella typhimurium*. The enzyme designators are: (1) L-aspartate (ASP) oxidase; (2) quinolinic acid (QA) synthetase; (3) quinolinic acid phosphoribosyl transferase; (4) nicotinic acid mononucleotide (NaMN) adenylyl transferase; (5) nicotinamide adenine dinucleotide (NAD) synthetase; (6) NAD kinase; (7) DNA ligase; (8) nicotinamide mononucleotide (NMN) deamidase; (9) NMN glycohydrolase; (10) nicotinamide (Nm) deamidase; (11) nicotinic acid (NA) phosphoribosyl transferase. DHAP, dihydroxyacetone phosphate; IA, iminoaspartate; PRPP 5-phosphoribosyl-1-pyrophosphate. The relationship of the known genetic markers is given.

## Materials and Methods

### Materials

[$^3$H]-labelled nicotinic acid and [$^{14}$C]-labelled adenine were purchased from Amersham-Searle, U.S.A. DEAE paper (Whatman DE81) was purchased from H. Reeve Angel Co, U.S.A.

### Culture media and growth conditions

E media of Vogel and Bonner (9) supplemented with 0.2% glucose was used as minimal media. Alternative carbon source was added to 0.2% in E media lacking citrate (2). All cultures were grown at 37℃. Aerobic liquid cultures were grown in an incubator rotary shaker (New Brunswick Scientific) at 200 rpm. Anaerobic cultures were grown in an anaerobic chamber (model 1024; Forma Scientific Co.) under an atmosphere of 3% hydrogen 7% carbon dioxide 90% nitrogen.

### Growth of cells in differentially labeled niacin and adenine

Cells were grown in 10 ml of a minimal medium containing high specific activity of [$^3$H] niacin. When the absorbance at 595 nm was between 0.5 and 1.0, 1 $\mu$mole [$^{14}$C]-labeled adenine (total radioactivity is 50 microcurie) was added.

The culture was allowed to grow for 0.5~1.0 hour in the presence of radioactive adenine, and the cells were harvested, centrifuged, washed with minimal medium, and transferred to 40 ml of a minimal medium which does not contain any radioactivity. Samples (12 ml) were taken for chromatographic analysis at various times after the shift to the new medium.

### Chromatography of pyridine nucleotides

The withdrawn samples (12 ml) from the culture were harvested by centrifugation at 8,000 rpm for 10 min in a Sorvall RC-2B centrifuge and washed with 0.5 M Tris buffer (pH 8). Cells were resuspended in 0.2 ml Tris buffer and HCl was added to final concentration of 0.15 M. Acidified samples were placed in a cold room overnight, and the debris was pelleted for 15 min in an Eppendorf centrifuge. The portion of supernatant (0.1 ml) was spotted on DEAE-paper with 0.02 ml of solution containing 5 mg/ml of NAD, NADP, nicotinamide as markers, and the chromatogram was developed with 0.25 M $NH_4HCO_3$ as a solvent for 6~8 hours. The chromatogram was dried, the spots were visualized, and 1 cm strips were cut and counted on the [$^3$H] and [$^{14}$C] channels of a liquid scintillation counter.

## Results and Discussion

### Breakdown rate of NAD under aerobic condition

The breakdown rate of NAD in *E. coli* has been measured before (7), but that of *S. typhimurium* has not been reported. Consequently, the rate of the NAD breakdown of wild type *Salmonella typhimurium* LT2 under aerobic conditions was measured.

In *Salmonella typhimurium*, almost all the nicotinamide

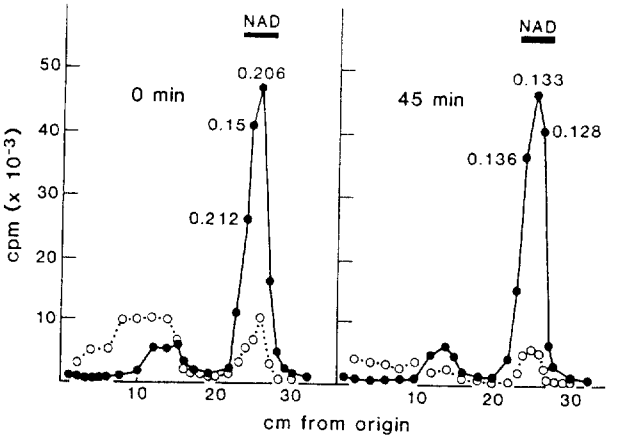


**Fig. 2.** Chromatographic analysis of radiolabel in the intracellular NAD peak at the time of labeling (left panel) and after 45 min chase. The [$^3$H] radioactivity (solid line (—) in the NAD peak which represents the amount of radiolabeled nicotinamide ring in the NAD pool does not significantly decrease with time; in contrast, the [$^{14}$C] radioactivity (dotted line(----)) which represents the remaining radioactivity in the adenine moiety of the original NAD pool shows an exponential decrease. The numbers beside each fraction along the NAD peak are the [$^{14}$C]:[$^3$H] ratio for that particular fraction. Solid line (—): [$^3$H]-nicotinamide radioactivity. Dotted line (----): [$^{14}$C]-adenine radioactivity.

moiety broken from NAD has proven to recycle back to NAD pool. In contrast, adenine containing moiety has very low probability of recycling because adenine could be used to make many other metabolites (7).

If we pulse label the endogeneous NAD with [$^{14}$C]-adenine, and follow the loss of [$^{14}$C]-adenine from NAD pool, we can basically measure the NAD breakdown rate. However, we radiolabel the nicotinamide containing moiety of endogeneous NAD, with [$^3$H] as well as adenine containing moiety with [$^{14}$C]. Since almost all the nicotinamide moiety recycle back to NAD (7), the amount of [$^3$H] can denote the amount of total NAD inside of the cells at each time.

LT2 cells were grown aerobically in a medium containing both [$^{14}$C]-adenine and [$^3$H]-niacin, and then shifted to a new medium which had no radioactivity. The portions of cultures were harvested and ruptured at various times. Then cell-free crude extracts were applied on DEAE chromatography paper, and endogenous NAD (the major peak indicated by arrow in Fig. 2) was isolated from other metabolites which might contain radioactivity, i.e., nicotinamide, NMN, NADP, AMP, ADP, ATP. All the radioactivity in the peak presumably comes from NAD molecules whose purity in this system has been proven before (7). The [$^3$H] radioactivity in the NAD peak which represents the amount of radiolabeled nicotinamide ring in the NAD pool does not significantly decrease with time; in contrast, the [$^{14}$C] radioactivity

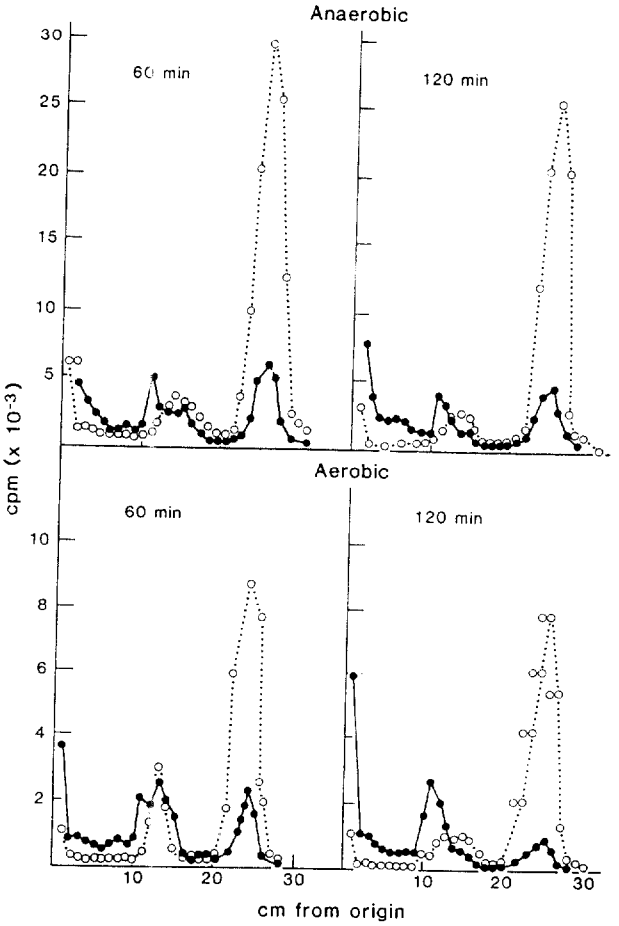


**Fig. 3.** Comparison of radiolabel in NAD & recycling pyridine nucleotide species between anaerobically grown cells in glucose supplemented medium (upper panel) and aerobically grown cells in glycerol supplemented medium (lower panel). It is clear that there has been much less loss of [$^{14}$C]-adenine radioactivity (solid line) in anaerobic than aerobic condition. Solid line (—): [$^{14}$C]-adenine radioactivity. Dotted line (----): [$^3$H]-nicotinamide radioactivity.

(which represents the remaining radioactivity in the adenine moiety of the original NAD pool) shows an exponential decrease. These two different behaviors of radioactivity were identical to that of *E. coli*.

[$^{14}$C]/[$^3$H] ratio at a certain time normalized with the ratio at zero time, represents the fraction of uncleaved NAD. The [$^{14}$C]/[$^3$H] ratio for each fraction along the NAD peak on the chromatogram was separately calculated and averaged at each time point (Fig. 2). NAD turnover thus calculated is less than 2 hours in *S. typhimurium*. This decay rate is similar to that of *E. coli*.

## Breakdown rate of NAD under anaerobic condition

In the next step, the breakdown rate of NAD in LT2 cells under anaerobic conditions was measured and compared with aerobic conditions. For measuring the anae-

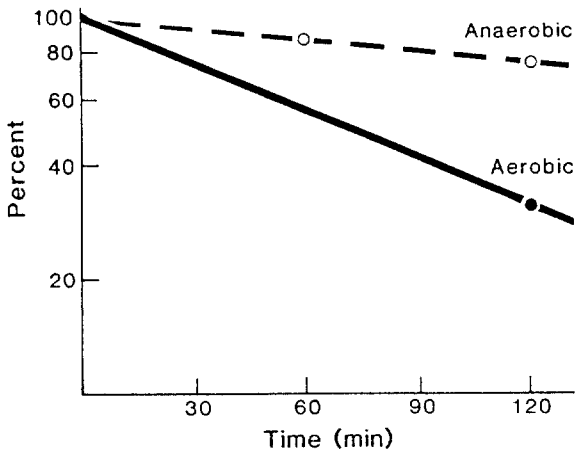


**Fig. 4.** Breakdown rate of NAD; aerobically versus anaerobically grown cells. The ratio of $^{14}C$ to $^{3}H$ is plotted as a function of time based on data such as is shown in Fig. 3. It is clear the decay rate is much faster in aerobic condition. Since both cultures have more or less same doubling time, oxygen seems to have important effect in breakage of NAD.

robic breakdown rate, LT2 cells growing in anaerobic chambers were used. The minimal medium was stored for several days inside the chamber and used for the anaerobic growth medium. After cells were grown in such an anaerobic medium containing [$^{14}C$]-adenine and [$^{3}H$]-niacin, as described in Materials and Methods, the anaerobic culture was moved from the anaerobic chamber and cells were quickly harvested. The cell pellet was brought back to the anaerobic chamber and resuspended in a new anaerobic medium which did not contain any radioactivity. Portions of the anaerobic culture were withdrawn at various time points, and the fate of endogenous NAD was followed (Fig. 3, upper panel).

The distribution of [$^{3}H$] is very similar to that in aerobic condition and the amount of [$^{3}H$] found in NAD peak has not been reduced significantly after 2 hours (Fig. 3, upper panel). This means almost all the nicotinamide moiety broken from NAD was recycled back to NAD pool. However, the amount of [$^{14}C$] found in NAD peak was not significantly reduced either. In aerobic condition, the half-life of NAD calculated by [$^{14}C$]/[$^{3}H$] ratio was less than 2 hours, however in anaerobic condition it was more than 6~8 hours.

In anaerobic conditions, cells grow slower compared to aerobic conditions. Difference in growth rate might affect the NAD breakdown rate. Therefore, as a control, the NAD turnover rate of an aerobic culture, which had approximately the same doubling time as the above anaerobic culture, was measured (Fig. 3 lower panel). This aerobic culture was supplemented with glycerol instead of glucose so that the cells grew more slowly. However, cells grown under aerobic conditions showed identical decay rates of NAD regardless of doubling time.

The comparison between aerobic and anaerobic breakdown rate is presented in Fig. 4. It is clear that under the anaerobic conditions, the NAD turnover rate is reduced to 1/3~1/4 that of the aerobic conditions. Thus, the NAD breakdown rate under anaerobic conditions seems to be reduced not because of the difference in doubling time, but because of the different metabolic state of the cell.

The results presented above indicate that the majority of NAD breaking activity takes place only in the aerobic condition. The finding that the major portion of NAD breakdown only occurs aerobically is an important one, since it suggests that majority of the turnover activity might be somehow linked to the activity which is boosted in the presence of oxygen, or somehow related to metabolism of oxygen. Several activities have proven to be activated in aerobic conditions. The cytochromes and other components of the respiratory system are greatly increased in aerobically grown facultative bacteria. Pyruvate dehydrogenase is induced around 4-5-fold in aerobic conditions and the TCA cycle is activated too (3, 5). We can imagine that the increased usage of the NAD under the aerobic condition, might result in the damage to the NAD molecule itself. The increased rate of NAD breakdown under the aerobic conditions might represent the scavanging activity of such damaged NAD.

The reactions which are related to signaling and/or repair of oxidation damage would operate constantly in the presence of oxygen regardless of growth rate or composition of medium. Partially reduced oxygen species like the superoxide radical or hydrogen peroxide, if they escape from dismutase or catalase can damage various components of the cell. Fluxes of superoxide and hydrogen peroxide, generated enzymatically or photochemically, have been shown to inactivate viruses, induce lipid peroxidation, damage membranes, cause single strand breakage and other damage to DNA. NAD might be used to repair one or more of the above disturbances.

Therefore, it is also an attractive hypothesis that major NAD turnover might be related to signaling or repairing of oxidation damage either to DNA (1) or to the membrane or to other cell components.

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