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OXYGEN POISONING OF NAD BIOSYNTHESIS: A PROPOSED SITE OF CELLULAR OXYGEN TOXICITY

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SUMMARY: Quinolinate phosphoribosyl transferase was rapidly inactivated in Escherichia coli exposed to hyperbaric oxygen. The enzyme is essential for de novo biosynthesis of NAD in E. coli and man. Because of its sensitivity and essentiality, inactivation of this enzyme is proposed as a significant mechanism of cellular oxygen toxicity. Niacin which enters the NAD biosynthetic pathway below the oxygen-poisoned enzyme provided significant protection against the decrease in pyridine nucleotides and the growth inhibition from hyperoxia in E. coli and could be useful in cases of human oxygen poisoning.

INTRODUCTION: Life forms which use oxygen must avert the potentially harmful effects of oxygen radicals which are formed by univalent reduction. Cells are protected from the toxic radicals by enzymes such as superoxide dismutases, catalases and peroxidases. The margin of safety is narrow, however, and aerobic life forms suffer damage in oxygen tensions elevated as little as 2 to 10 times that of air [1, 2].

Using Escherichia coli as a model for some aspects of cellular oxygen toxicity, we obtained partial protection with specific amino acids [3]. Certain intermediates of the biosynthetic pathways for these amino acids also protected, indicating that specific enzymes for synthesis of branched-chain and aromatic amino acids were inactivated rapidly upon exposure of E. coli to hyperbaric oxygen [3]. Subsequently, dihydroxyacid dehydratase was identified as the specific site of oxygen poisoning in biosynthesis of branched-chain amino acids [4]. These data accounted for the acute growth-cessation in minimal medium as the consequence of inhibition of protein synthesis and induction of stringency through deprivation of specific amino acids. However, the protection afforded by these specific amino acids decreased on continued exposure to high oxygen tension [3]. This might be expected if oxygen inhibited synthe-

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sis of other essential components which are consumed more slowly during growth than are amino acids. In this paper we report evidence that inhibition of the niacin-NAD biosynthetic pathway is one such additional site of oxygen poisoning.

MATERIALS AND METHODS: Conditions for measurement of growth of bacteria in hyperoxia in medium supplemented with various intermediates were as previously described [3]. Escherichia coli K-12 mutant strains were obtained from Gholson [5]. Cells were exposed to hyperoxia, enzyme extracts were prepared and protein was measured as previously described [4].

Crude extracts and the 35% ammonium sulfate supernatant were assayed for quinolinate phosphoribosyl transferase using quinolinate-2,3,7,8 (-^{14}C) synthesized as previously described [6]. The reaction mixture (0.4 ml) contained: 0.5 µmole ^{14}C-quinolinic acid (190 µCi/mmole), 20 µmoles potassium phosphate buffer, pH 7.1, 2 µmoles Mg acetate, 0.12 µmole PRPP and enzyme extract (3-10 mg protein). Incubation was for 60 min at 30°C in special flasks (Kontes #K-882350) with attached scintillation vials [7]. The reaction was terminated with 0.5 ml of 5 N H_2SO_4 and the $^{14}CO_2$ evolved was trapped in 0.5 ml of phenethylamine methanol (1:2 v/v) and counted by scintillation spectrophotometry. One nmole of CO_2 gave 105 DPM. Under assay conditions, quinolinate decarboxylation is linear for 1 hr and proportional to enzyme concentration up to 10 mg crude protein/assay. One unit of enzyme converts 1 nmole of quinolinate to CO_2 and nicotinate mononucleotide per min.

Phosphoribosylpyrophosphate synthetase was measured by a modification of the two part assay of Kornberg et. al [8]. The reaction for part one (1.1 ml) contained: 66 µmole potassium phosphate buffer, pH 7.4, 2 µmole ATP, 4 µmole ribose-5-phosphate, 10 µmole $MgCl_2$, 10 µmole dithiothreitol and 10-20 µl of crude enzyme extract (0.2 to 0.4 mg protein). A reaction with ATP and ribose-5-phosphate omitted was used as the blank. Following incubation for 10 min at 37°C, the reaction was stopped by heating at 100°C for 30 sec and cooling immediately in an ice-acetone bath. Precipitated protein was removed by centrifugation at 10,000 for 10 min. The reaction for part two (0.55 ml) contained: 11 µmoles tris-HCl, pH 8.0, 0.68 units of orotidine pyrophosphorylase-decarboxylase, 1.1 µmole $MgCl_2$, 0.3 µmoles [caroboxyl-^{14}C]-orotic acid (0.5 µCi/µmole) and 100 to 300 µl of reaction supernatant). The reaction was started by addition of radioactive orotate and after 60 min at 37°C was terminated by injection of 0.1 ml 5N H_2SO_4 through a rubber stopper. CO_2 was trapped and radioactivity was determined as described for the quinolinate phosphoribosyl transferase assay. Tests were done to arrive at the above substrate and enzyme concentrations, and incubation durations as within the linear limits of the assay. One mU of enzyme produced one µmole of CO_2 per hour.

The intracellular pool of phosphoribosylpyrophosphate was determined using the reaction for part two of the assay [8]. Crude extract was heated at 100°C for 60 sec and immediately cooled in an ice-acetone bath. Protein was removed by centrifugation and 0.2 ml of the supernatant was assayed as described above except that 0.1 mM [carboxyl-^{14}C]-orotic acid (5 µCi/µmole) was used.

For determination of pyridine nucleotides, cells were grown and exposed to hyperbaric (4.2 atm) of oxygen as described previously [4]. Cells were harvested through a cooling coil which reduced the temperature from 37°C to 4°C in approximately 1 min. Cells were extracted in 0.5N perchloric acid to preserve the oxidized coenzymes and in 0.5 N KOH in ethanol:water (1:1 v/v) to preserve the reduced coenzymes [9]. The conezymes were measured by a sensitive polarographic recycling assay [10].

RESULTS AND DISCUSSION: Five vitamins and parahydroxybenzoic acid (PHBA) were tested for their ability to protect E. coli from the toxic effects of 4.2 atm

Table 1 Protection Against Oxygen Toxicity by Niacin and Thiamine

Nutritional Status[a]	Generation time[b] (min) in 1 atm air	4.2 atm oxygen	
	--	0-2 hr	2-4 hr
Medium A	30±1.2	97±7.0[c]	4797±1781[c]
Medium B	25±0.4	47±1.3	112±3.2
Medium B minus			
Niacin	27±0.6	77±5.0[c]	803±188[c]
Thiamine	27±0.5	63±0.3[c]	162±3.6[c]
PHBA	27±0.6	52±1.5	--
Biotin	27±0.7	49±0.9	97±1.8
Pantothenate	27±0.6	49±0.7	105±2.7
Folate	27±0.5	51±0.7	104±2.5
PHBA, biotin, pantothenate, folate	27±0.6	52±0.7	114±6.2
Thiamine, niacin	26±0.3	97±1.0[c]	1030±180[c]
Valine	26±1.3	1242±215[c]	2884±352[c]
Medium B plus			
Pyridoxine	25±1.6	50±0.6	98±2.7
Riboflavin	27±0.3	49±0.8	104±5.4
PABA	26±0.4	52±0.9	103±3.9
α-Ketoisovalerate	25±0.7	52±0.7	114±2.4
Adenine, thymine, guanine, cytosine, uracil	24±0.4	51±0.3	144±4.8

[a] Medium A contained basal salts, 27.7 mM glucose and twenty amino acids (each at 0.65 mM) as previously described [3]. Medium B = medium A plus thiamine, biotin, pantothenate and folate, 0.1 mM each and PHBA and niacin, 0.2 mM. Compounds were deleted or supplemented (each at 0.1 mM except bases, 0.5 mM), as indicated. Cultures at an absorbance of 0.2 to 0.3 at 500 nm in exponential growth were pressurized with 1 atm of air plus 4 atm of oxygen (4.2 atm total oxygen pressure).

[b] Averages ± S.E.M. for 8 or more replicate cultures. Generation time becomes infinite as growth rate (Δ absorbance) decreases to zero; hence, the large values and standard deviations as growth decreased. For example, with valine omitted, the average change in absorbance over the final 2 hr in hyperoxia was only 0.007 absorbance.

[c] Significantly different ($p \leq 0.05$) compared to growth in Medium B, using Student's t-test.

of oxygen (Table 1). These supplements were added to basal salts medium containing glucose and twenty amino acids, a medium which previously gave significant protection for approximately one generation [3]. With niacin, PHBA, thiamine, biotin, pantothenate, and folate all present, cells grew considerably more rapidly in 4.2 atm of oxygen than did cells in medium without these com-

pounds (Table 1). This was evident during the first 2 hr of incubation (Table 1) in hyperoxia, and the difference increased on further incubation (2-4 hr interval). The inhibition of growth was caused by elevated oxygen tension and not by pressure, per se, since mixtures of air plus helium or nitrogen at the same total pressure did not inhibit (data not shown). Deletion of these compounds (Table 1) singly and in combinations indicated that only thiamine and niacin were protective, as evidenced by significantly decreased growth ($p \leq 0.05$) upon their deletion. When valine was omitted as a control (Table 1) results were similar to those previously reported [3].

Additional vitamins, bases, and other metabolites which were tested, failed to increase the protection when they were added to medium containing the protective amino acids [3] plus the protective vitamins thiamine and niacin (Table 1). To minimize the possibility that failure of a compound to protect against oxygen toxicity resulted from failure of transport, cells were grown for two subcultures in medium containing the test compound to preadapt them.

The best protective medium (containing amino acids, niacin and thiamine) gave significant growth in hyperoxia compared to basal medium alone or such medium supplemented with the amino acids found to be protective (Fig. 1). In the protective medium at 4 hr, growth was continuing with a constant generation time of approximately 104 min which was established after 2.5 hr of hyperbaric oxygen; without vitamins, essentially no growth was obtained after 70 min in hyperbaric oxygen (Fig. 1). The culture in medium with vitamins and amino acids continued to grow in hyperoxia (not shown) and reached an absorbance of 8.0 which was comparable to that obtained by the control with air as the gas phase (Fig. 1). Thus the protection afforded by amino acids, thiamine and niacin was sufficient to indicate that damage by hyperoxia to other systems must be comparatively much less significant to the cell.

The protective role of niacin was investigated further. Niacin contributes to the biosynthesis of nicotinamide adenine dinucleotide (NAD) via a

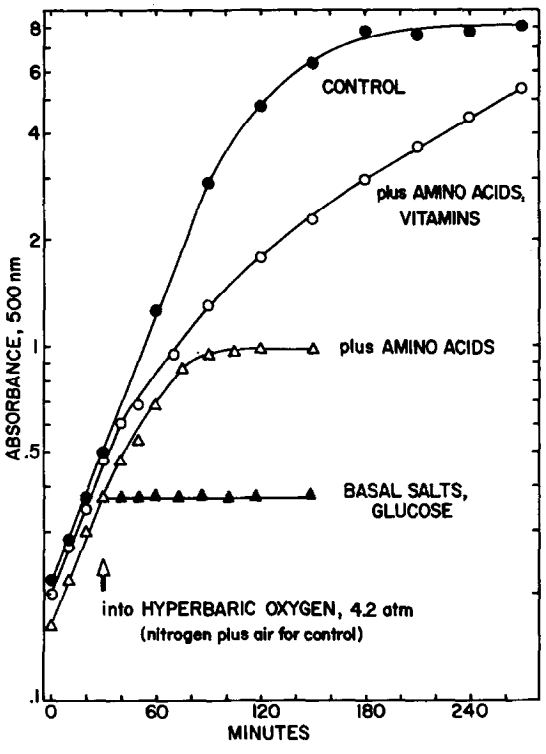

Fig. 1. Protective effects of amino acids and vitamins for growth of Escherichia coli in 4.2 atm of oxygen. Strain E-26 was grown in a stirred vessel with air as the gas phase. Cultures were pressurized with 4 atm overpressure of oxygen (gas phase, 1 atm of air plus 4 atm of oxygen) at 30 min in minimal basal salts plus glucose meduim [3] (▲), and the same medium supplemented with 20 amino acids [3], each at 0.65 mM (Δ), or 20 amino acids plus niacin and parahydroxybenzoic acid (PHBA), each at 0.2 mM, and pantothenate, biotin, folate, and thiamine, each at 0.1 mM (o). Cultures in the medium containing amino acids, vitamins and PHBA were also exposed continuously to air as a control (●). The same results were found (not shown) for the 10 amino acids previously found to protect [3] plus niacin and thiamine as is shown for 20 amino acids plus 5 vitamins and PHBA (o). Data were obtained by removing samples at intervals through a valve without decompressing the parent culture. Absorbances were measured at 500 nm wavelength. The relationship between absorbance and plate colony counts is linear up to 0.8 A; all measurements were made below 0.8 A by dilution. There are approximately 3×10^8 cells per ml per 1.0 A.

salvage pathway. Therefore, various other intermediates of NAD biosynthesis were tested for their ability to protect E. coli from the growth-inhibiting effects of 4.2 atm of oxygen. Growth was enhanced by each of the intermediates: mononucleotide (NMN), NAD, nicotinamide, and niacin; (Table 2). Quinolinate did not help in the first hr and was only marginally helpful over the next 2 hr (Table 2). The enhanced growth rate with the other intermediates was apparent after 2 hr and increased further over the interval 2-4 hr with all

Table 2 Inhibition of NAD Biosynthesis by Oxygen

The growth and analysis conditions and the basal medium were the same as described for Table I medium A, except that the indicated compounds (0.1 mM) were substituted for niacin. NMN is nicotinamide mononucleotide, NAD is nicotinamide adenine dinucleotide, and PRPP is 5-phosphoribosyl-1-pyrophosphate.

Compound Added	E. Coli Strain	Generation time (min)[a] 1 atm air --	4.2 atm oxygen 0-2 h	4.2 atm oxygen 2-4 h
Quinolinate	E-26	26±0.8	80±2.7	383±26
NMN	E-26	27±0.4	52±0.4[c]	190±12[c]
NAD	E-26	29±0.8	56±0.4[c]	167± 4[c]
Nicotinamide	E-26	26±0.4	53±0.4[c]	128± 2[c]
Niacin	E-26	25±0.4	47±1.3[c]	112± 3[c]
None	E-26	27±0.6	77±5.0	803±188[c]
Quinolinate	K-12(W3899)[b]	35±0.6	85±1.0	241± 3
Niacin	K-12(W3899)	36±0.9	56±1.7[c]	119± 4[c]
Quinolinate	K-12(W3899-N)[b]	35±0.7	78±0.9	262±17
Niacin	K-12(W3899-N)	33±0.5	52±0.6[c]	118± 2[c]
Quinolinate plus PRPP	K-12(W3899-N)	34±0.1	94±2.2	180± 4[c]

[a]Averages ± S.E.M. for 8 or more replicates analyzed as described in Table 1.

[b]Mutant strain sources identified in text.

[c]Significantly different ($p \leq 0.05$) compared to growth for the strain with quinolinate, using Student's t-test.

strains (Table 2). Tests with the critical intermediates were confirmed (Table 2) using two mutant strains (W3899 and W3899-N) which are known to transport quinolinate since they grow with quinolinate substituted for niacin [5]. Strain W3899 will grow with niacin, nicotinamide, NAD and NMN as a source of the pyridine ring [5]. Strain W3899-N will grow with niacin, NMN or NAD, but not with nicotinamide [5]. NAD is not used, *per se*, but enters *via* the salvage pathway after partial degradation [5]. PRPP (5-phosphoribosyl-1-pyrophosphate), when added with quinolinate had little measurable effect in this test (Table 2). All organisms were preadapted by growth in medium containing the test compounds. However, PRPP is acid and temperature unstable and degrades readily.

The pattern of protection shown by intermediates in Table 2 suggests a specific site of inhibition by hyperoxia. The simplest interpretation of the

Table 3. Effects on Enzyme Activities and PRPP Intracellular Pool of One Hour Exposure of Escherichia coli to Hyperbaric Oxygen[a]

	Specific Activity (mU/mg)			
	Quinolinate Phosphoribosyl Transferase			
Gas Phase	Crude Extract	$(NH_4)_2SO_4$ fraction	PRPP Synthetase	PRPP Pool[b]
Air	41.9±2.11 (5,20)	43.1±3.43 (2,8)	15.6±3.3 (2,15)	$4.91\pm0.48\times10^3$ (3,12)
HPO	3.3±0.31 (3,8)[c]	3.2±0.23 (2,7)[c]	15.1±2.0 (2,15)	$3.55\pm0.67\times10^4$ (3,12)[c]

[a] Escherichia coli K-12 was grown at 37°C in medium A (Table 1) which contained amino acids to prevent growth inhibition due to poisoning in the amino acid biosynthetic pathways [3]. HPO = 1 atm of air plus 4 atm of oxygen (4.2 atm O_2). The enzyme assay procedures and definitions of units of activity are given in Materials and Methods. Averages ± S.E.M. are shown with the number of experiments and total determinations in parenthesis.

[b] Molecules/cell.

[c] Significantly lower ($p \leq 0.001$) compared to air controls, using Student's t-test.

increased growth rate achieved by inclusion of each of the intermediates which lie beyond quinolinate in the pathway of NAD biosynthesis is that exposure of cells to hyperbaric oxygen blocks the synthesis of nicotinate mononucleotide by poisoning quinolinate phosphoribosyl transferase. Table 3 shows the inhibitory effect on quinolinate phosphoribosyl tranferase of exposure of exponentially growing E. coli to 4.2 atm of oxygen.

The specificity of the site of oxygen poisoning in NAD biosynthesis was examined further by measuring the activity of another enzyme in the pathway, phosphoribosylpyrophosphate (PRPP) synthetase. This enzyme produces PRPP which is required in two places for NAD biosynthesis. Poisoning of this enzyme would lead to a decreased pool of PRPP but would be circumvented by inclusion of any of the intermediates (NMN, NAD) containing the phosphoribose moiety (Table 2). Table 3 shows that phosphoribosylpyrophosphate synthetase was not decreased in specific activity in cells poisoned by hyperoxia. The intracellular pool of PRPP, however, increased seven-fold (Table 3). This PRPP increase is consistent with the oxygen poisoning of quinolinate phosphoribosyl transferase

since this enzyme uses PRPP as a substrate for synthesis of NMN from quinolinate. A preliminary report from our laboratory [11] indicating oxygen poisoning of PRPP synthetase has been determined to be in error because the assay is strongly inhibited by the large amount of crude extract (14 mg protein) which was used previously [11] in the assay for oxygen-exposed cells.

Another indication of the specificity of the oxygen poisoned site and evidence of its biological signifiance was obtained by comparing the total pyridine nucleotide (NAD, NADH, NADP, NADPH) content of cells grown under different nutritional states with air as the gas phase and cells poisoned in 4.2 atm of oxygen (HPO). The total pyridine nucletodide content in thousands of molecules per cell were as follows: without niacin: in air, 1726±257; in HPO for 1 hr, 637±154; with 0.2 mM niacin: in air, 3327±298; in HPO for 1 hr, 3573±118; with 0.1 mM quinolinate substituted for niacin: in air, 1900±26; in HPO for 1 hr, 650±176. These data show that the pyridine nucleotide content of cells decreased significantly at $p \leq 0.001$ after 1 hr in HPO and that this drop was prevented by niacin but not by quinolinate.

Oxygen poisoning of quinolinate phosphoribosyl transferase would have serious consequences for E. coli. Because the biosynthetic pathway of NAD beyond quinolinate is the same in man and other mammals as in E. coli [6,12,13], the findings appear to have general relevance to oxygen toxicity. Without this enzyme, NAD synthesis would cease when the supply of intermediates, including niacin from the salvage pathway, was depleted. NAD and NADP are coenzymes for over two hundred biochemical reactions and, it has been shown recently that NAD is consumed by major cellular reactions including cleavage of NAD to nicotinamide with formation of polyadenine diphosphoribose in eucaryotes [14-17] and cleavage to form AMP and NMN by bacterial ligases [18, 19]. The half-lives of NAD are reported to be approximately 2 hr in E. coli and in animals [20-22], and approximately 1 hr in cultured human cells [23,24]. Approximately 95% of the synthesized NAD is consumed by catabolic reactions in the nucleus of mammalian cells and only 5% is used to maintain the NAD co-

enzyme pool [24]. Blockage of NAD synthesis by hyperoxia, due to the fact that NAD is consumed and has a comparatively short half-life, would result in proportionately rapid onset of serious metabolic defects. Therefore, it appears that the findings may be relevant to the use of oxygen for therapy of human conditions such as gas gangrene, prematurity, carbon monoxide poisoning, aeroembolism and cardiovascular and lung dysfunctions where oxygen toxicity is a limitation.

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