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MECHANISMS OF OXYGEN TOXICITY AT THE CELLULAR LEVEL.(U)
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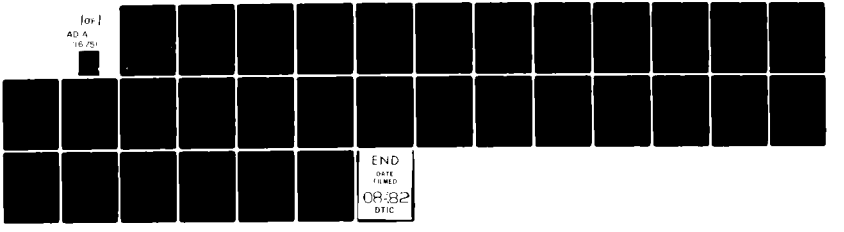

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FINAL REPORT

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Mechanisms of Oxygen Toxicity at the Cellular Level

By

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FINAL REPORT

#39, June 30, 1982

(Covering the Period 1-1-68 to 9-31-81)

PROJECT TITLE:

"MECHANISMS OF OXYGEN TOXICITY AT THE CELLULAR LEVEL"

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and the Department of Veterinary Microbiology

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Assisted by: Twenty-four individuals (see section V)

WORK UNIT NO. NR204-020 CONTRACT: N00014-76-C-0328

This report consists of the following: (I) Summary of Significant Results, (II) Summaries of Results by Research Topics, (III) Conclusions, (IV) Major Accomplishments, (V) Technicians and Students who Received Training and Support, (VI) Index of Reports, Including Annual Reports, (VII) Index of Publications, and Distribution List and DD form 1473.

(I) SUMMARY OF SIGNIFICANT RESULTS

Except for radiation sensitive strains which are killed by hyperoxia, the effect of hyperoxia on bacteria is principally bacteristatic. Hyperoxia produces a reversible inhibition of growth, respiration, and fatty acid synthesis. The interruption of fatty acid synthesis is not the result of inactivation of the fatty acid synthetase enzyme complex, nor is it due to a lack of reduced pyridine nucleotide coenzyme (NADPH) but results from stringency induction as detailed below.

Studies involving a nutritional approach using vitamins, precursors, and intermediates with analysis of carbohydrate, amino acid, lipid, protein, RNA and DNA synthesis and direct analysis of specific enzymes in suspected pathways

have provided data to support the following sequence of events during oxygen intoxication of Escherichia coli. Oxygen inhibits biosynthesis of several identified amino acids. Within less than 5 minutes the intracellular concentration of valine is limiting for protein synthesis (subsequently 9 other amino acids become limiting if valine is supplied). The unloaded tRNA's also stimulate the "stringent response" resulting in production of a powerful inhibitor (guanosine tetraphosphate) of various processes including: transport, and synthesis of RNA, lipids and others. This completely stops most biosynthetic events when the cells are exposed to hyperbaric oxygen in medium without the critical amino acids.

When the amino acids are present, protection from the stringent response occurs. However, quinolinate phosphoribosyl transferase, an enzyme required for de novo synthesis of NAD, also is oxygen-sensitive. As intracellular dilution of coenzyme occurs due to growth and because of very active catabolic processes (the half-life for NAD is reported to be 2 hours), the concentrations of the coenzymes become limiting for the probably more than 200 reactions dependent on NAD. Niacin, a B-complex vitamin which enters this biosynthetic pathway below the poisoned enzyme, prevents the inhibition of coenzyme biosynthesis and reduces the growth inhibition. Thiamin (a B-complex vitamin) further reduces the growth-inhibition caused by hyperoxia, and thiamin concentration is rapidly and significantly reduced during oxygen poisoning. Whether thiamin is decreased by inhibition of synthesis or by destruction (as by oxidation to thiochrome) was not proven. However, thiamin intermediates protect like thiamin. Thus, hyperoxia inhibits reactions requiring thiamin pyrophosphate which include: synthesis of branched-chain amino acids, the pentose shunt pathway which provides NADPH needed for reductive biosynthsis and intermediates for nucleic acid and aromatic amino acid synthesis and the decarboxylation-dehydration reactions of αketoglutarate and pyruvate.

A combination of different bacterial strains, nutritional conditions and chloramphenicol was used to suppress or allow stringency and the consequences of known sites of oxygen toxicity, and the effects of hyperbaric oxygen were measured on net synthesis by Escherichia coli of RNA, DNA, protein and lipid. With stringency mitigated and optimal nutritional protection, instead of stasis there was a reduction of growth and net synthesis in hyperbaric oxygen. With such conditions, however, the net synthesis of RNA, DNA, and protein occurred in approximately balanced amounts as determined by comparison with net synthesis by control cells with air as the gas phase. In medium without protective amino acids, induction of stringency was a principal indirect cause of inhibition of net synthesis of RNA and lipid. However, there also was evidence of significant direct impairment of RNA net synthesis. Net synthesis of DNA was reduced either because of an effect directly on DNA metabolism or indirectly because of impaired RNA synthesis. Protein net synthesis was stopped indirectly when amino acids, whose syntheses are known to be poisoned in hyperoxia, were omitted from the medium. This occurred indirectly via the stringency control mechanism and due to the lack of specific amino acids. However, with amino acids provided and stringency prevented there still was a small amount of impairment of protein synthesis in hyperoxia. This may have occurred via the observed net inhibition of RNA synthesis. Plasmolyzed cells, prepared after oxygen poisoning, showed impaired RNA and DNA net synthesis capability from nucleotides, indicating that inhibition occurred subsequent to nucleotide synthesis.

The enzymes of glycolysis, Krebs cycle and oxidative phosphorylation, and the transport machinery appear to be relatively resistant and remain functional

for up to 4 generations (the maximum tested to date). ATP deficiency is not the cause of growth inhibition, neither is growth inhibition caused by an overall change in oxidation state of the cellular SH groups.

Several of these sensitive sites (including quinolinate phosphoribosyl transferase, required for NAD synthesis) should be relevant to higher systems, including man. The data suggest that the vitamins thiamin pyrophosphate and niacin should be tested as potential protective agents against the toxicity in man of oxygen and of chemicals which produce cellular oxidative stress.

(II) SUMMARIES OF RESULTS BY RESEARCH TOPICS

(1) LETHALITY OF CO_2-DEPRIVATION

A membrane-culture technique was developed for directly exposing bacteria to hyperoxic atmospheres and quantifying the fate of individual cells. Using this procedure, the effect of pure oxygen at one atmosphere upon various species of bacteria growing on nutrient agar (Difco) was tested. Results obtined were as follows. CO_2 is essential to prevent death of Escherichia coli, and probably other bacteria, if the cells are provided with an otherwise adequate environment for growth. With low population densities, oxygen, nitrogen, or CO_2-free air at one atm is bactericidal for E. coli through the indirect mechanism of deprivation of CO_2; e.g., addition of 0.03% CO_2 gives complete protection. With population densities above about $10^7/\mu^2$ on Millipore membranes, the CO_2 requirement is supplied by the cells. Large inocula survived and were protective for small inocula which were physically separated except for gaseous diffusion by placing the two membrane cultures a few millimeters apart and directly opposite each other. This protective effect was abolished by presence of a NaOH trap between the two cultures. The death rate of E. coli in oxygen at 1 atm. with population densities below about $10^3/\mu^2$ closely approximately first-order reaction kinetics with temperature-dependent half-lives ranging from 148 to 61 minutes when exposed on nutrient agar over the temperature range 26 to 40°C. Below 16°C, a point at which growth potential ceases, no killing was obtained. On synthetic media with a single carbon source (glucose or acetate), protection resulted (half-life greater than 24 hrs). Exposure to oxygen at 37°C in the absence of organic nutrients produced no killing. The results indicate that killing due to lack of CO_2 in a pure oxygen environment is dependent upon metabolic or growth-rate potential. The μ value (the biological counterpart of the energy of activation) for death of E. coli in O_2 at 1 atm. in the absence of CO_2 was about 10,000 calories/degree over the range 26-40°C. The value increased rapidly below 24°C and approached infinity below 15°C. This behavior is similar to other biological processes which are enzyme catalyzed.

Oxygen at 1 atm. with added CO_2 equivalent to that in air gave little measurable effect upon growth rate, or rate of acetate utilization as measured by oxygen uptake, or composition of fatty acids as determined by gas chromatography, but a slight depression in sulfhydryl concentration/cell was detected.

The effect of an environment of 1 atmosphere of air plus 4 atmospheres over-pressure of pure oxygen using E. coli, strain E-26 growing on synthetic medium with acetate as the carbon source was studied. A bacteristatic effect was obtained with no measurable growth, but no significant killing (similar results were obtained with 15 atmospheres overpressure of O_2 and 1 atm

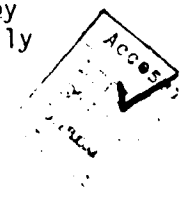
of air to provide CO_2). Upon removal of cells to air, growth occurred after a lag period.

To futher pursue this finding, Escherichia coli, Aerobacter aerogenes, and Staphylococcus albus were exposed to various gases using the membrane-culture technique modified as described here. Bacteria in log phase growth were washed by centrifugation and resuspension in potassium phosphate buffer, pH 7.0, 0.05 M. Five replicate membrane cultures were prepared for each condition by filtering 10 ml of suspension diluted to contain a few hundred bacteria. Cultures were exposed at 37°C in incubators which were evacuated to 80 torr and refilled three times with experimental gas. Cultures were reincubated in air for 18 hours, and colonies were counted. Carbon dioxide-deprivation was achieved by a combination of methods: (a) adjusting media to pH 6.7, (b) the inverted half of each petri dish culture contained filter paper saturated with 1 N KOH, (c) gases were bubbled through spargers submerged in 2 traps (in series) each containing one 1 of 1 N KOH, and (d) before adding cultures, incubators (which contained filter paper saturated with 1 N KOH) were purged for 20 min.

Carbon dioxide deprivation had a comparable lethal effect on A. aerogenes and E. coli and pure O_2 and CO_2-free air produced similar killing. S. albus had less requirement for exogenous CO_2, and CO_2 deprivation produced only slight toxicity. Addition of 30 mm CO_2 stopped the killing. The TCA cycle intermediates, acetate and malate (which probably increased intracellular production of CO_2) reduced the toxicity for Escherichia coli. The absence of a nitrogen source (which prevented growth) also greatly reduced the toxicity of CO_2 deprivation.

These data indicate that bacteria differ in their CO_2 requirements, and CO_2 may or may not be adequately supplied by metabolism or the gas phase, depending on conditions. Knowledge of the bactericidal effect of CO_2 deprivation is relevant for research with bacteria exposed to pure gases which can lead to CO_2 deprivation. For example, in one of our publications (1) and in other work [see (2) for a review] the toxicity of pure O_2 most probably resulted from CO_2 deprivation. Also, if CO_2 is not purposely included, variable results may occur from contamination with CO_2 from the air.

Carbon dioxide is required by heterotrophs for synthesis of aspartate, glutamate, arginine, pyrimidines, purines, and lipids. Interruption of these reactions (or unknown CO_2-requiring reactions) appears responsible for CO_2-deprivation killing under conditions which otherwise permit growth. It has long been known that CO_2 deprivation is bacteristatic for some microorganisms, and for optimum growth more CO_2 than is present in air may be required. In 1968 it was shown that CO_2-deprivation causes lysis of several facultative microbes growth anaerobically but not aerobically (3). Our finding that CO_2-deprivation is bactericidal for Escherichia coli grown aerobically was demonstrated because dispersed bacteria were incubated on membranes where colony development from single cells could be quantified and the effect of metabolically produced CO_2 was minimized. [Refer to references 1, 2, 3, 4, 5, 19, 30].



(1) Brown, O.R. and Huggett, D.O. App. Microbiol. 16:476-79 (1968).

(2) Gottlieb, S.F. Ann. Rev. Microbiol. 25:111-52 (1971).

(3) Choe, B.K. and Bertani, G.J. Gen. Micorbiol. 54:59-66 (1968).

(2) CORRELATIONS BETWEEN SENSITIVITY TO RADIATION AND TO HYPEROXIA

A correlation was discovered between the sensitivities of Escherichia coli strains to radiation and to hyperoxia. Over the region from 1 to 15 atm of oxygen, percentage survival was minimal for all strains at 4.2 atm of O_2 (strain E-26, 51%; B, 1.5%, B/r, 60%; and B_{s-1} 0.25%). We concluded that strains B and B_{s-1}, which are radiation sensitive, were also more sensitive to the bactericidal effect of hyperoxia than was strain B/r, which is radiation resistant.

The trend toward correlation between sensitivity to radiation and sensitivity to hyperoxia was investigated further. E. coli K-12 polymerase + (resistant to X-ray) and polymerase negative (sensitive to X-ray) strains were both relatively resistant to hyperoxia but on the average, the strain lacking polymerase was approximately 1.4 times as sensitive to hyperoxia over the range, 2.2 atm to 15.2 atm O_2. This suggests that single-stranded breaks in DNA (repairable by polymerase) may have some role in oxygen toxicity. Salmonella typhimurium KSU strains 3845 and 2480 (sensitive to UV and X-rays) showed statistically significant killing at 4.2 atm of oxygen while 3598 and 2484 (sensitive to UV, resistant to X-ray) did not. No strains, whether resistant or sensitive to X-, γ or UV radiation, approached the sensitivity of B_{s-1} γR (a mutant reported to be resistant to γrays but not to UV) which was at least 100 times as sensitive at 4.2 atm of oxygen. The correlation between sensitivity to radiation and to hyperoxia previously found for B strains of E. coli, does not appear to be a universal property of radiation-sensitive bacteria. [Refer to references 11, 14, 18, 21, 22.]

(3) RELATIONSHIPS BETWEEN SULFHYDRYL AND OXYGEN TOXICITY

Cell-free extracts of Escherichia coli, grown in minimal medium with air as the gas phase, contained 26.8 $\pm$ 1.2 nmoles of sulfhydryl (SH) per mg of soluble protein (4.71×10^6 SH per cell). Approximately 92% of the SH groups were protein-related. The number of SH groups detected on the bacterial surface was $2.22 \pm 0.19 \times 10^5$ per cell. Exposure of intact bacteria to 1 atmosphere absolute of oxygen, which reduced the growth rate, or to 6.2 atm of oxygen which stopped growth almost instantaneously, did not significantly alter the SH concentration measured in cell-free extracts. However, SH groups were oxidized at approximately 0.5% per minute in cell-free extracts which were exposed to 1.2 atm of oxygen. SH groups detectable on the bacteria surface, were oxidized relatively slowly at approximately 0.2% per min at 6.2 atm of oxygen. We concluded that oxygen toxicity in this system was not caused by the mechanism of a generalized oxidation of SH groups. The data do not rule out the possibility of a mechanism of toxicity through oxidation of a relatively small number of critical SH groups in essential molecules, as in the membrane, for example.

Reduced lipoate (sodium salt of DL-6, 8-thioctic acid) at 10^{-3}M with air as the gase phase was not measurably toxic for growth or respiration of Escherichia coli; 5×10^{-2}M and higher concentrations were inhibitory. Bacteria were shown to be capable of oxidizing exogenously supplied reduced lipoate. Reduced lipoate (10^{-3}M) present prior to, during, or following exposure to hyperbaric oxygen, did not protect cells from the inhibition of growth and respiration produced by hyperbaric oxygen with either glucose or acetate as the carbon source. The normal rate of recovery of cell growth which occurred upon removal of bacteria from hyperbaric oxygen was delayed by the addition of

10^{-3}M reduced lipoate; this inhibition was possibly due to the production of toxic concentrations of oxidized lipoate by hyperic oxygen exposure. The minimal inhibitory concentration of added oxidized lipoate was between 1 and 5 x 10^{-3}M. [Refer to references 17, 23, 28.]

(4) EVALUATION OF THE EFFECTS OF HYPERBARIC OXYGEN IN VITRO AND IN VIVO ON COMPOSITION AND SYNTHESIS OF FATTY ACIDS.

Synthesis by Escherichia coli of straight-chain fatty acids was logarithmic in air-exposed controls, but was completely stopped in oxygen under the above conditions. Upon removal to air, synthesis of fatty acids resumed after a lag period. This reversible inhibition by oxygen occurred at pressures from 2 to 4 atm. O_2 at 1 atm or pressure per se (nitrogen) did not inhibit. The extent of inhibition was greatest at 4 atm but recovery time was longer at lower pressures where some fatty acid synthesis continued in O_2. Fatty acid synthetase specific activity in cell-free extracts was the same for air grown cells and for cells exposed for 3 hrs to 1 atm air plus 4 atm O_2 (0.18 nmoles malonyl CoA incorp. into fatty acids/min/mg protein). Composition of fatty acids, determined by gas chromatography, was not significantly different for cells grown in air, for cells exposed to O_2 at 4 atm, or for cells after reincubation for various intervals in air.

Exposure to O_2 at 15 atm for 2 hr resulted in an 80% reduction in respiration rate, using acetate grown cells with acetate as substrate for determination of O_2 uptake. The depression was reversed upon return of the cells to air and the recovery occurred in the absence of a nitrogen source. It appears that neither growth nor protein synthesis is required for recovery. The depression and recovery was similar for cells grown and measured with palmitate, but recovery was greatly delayed for cells grown, exposed and measured using glucose as the sole carbon source. Addition of SH containing reducing agents (cysteine, lipoic acid or dithiothreitol) before, during or after exposure to O_2 did not affect the rate of respiration inhibition or rate of recovery. The specific activity of thiolase was 319±135 for 19 determinations of air grown cells and 274±150 for 12 determinations of cells exposed to 1 atm of 95:5 (O_2:CO_2) plus 15 atm O_2. Five different cultures were measured and the deviation of individual cultures was less, being about 10%, and not significantly different for most of the experiments. Specific activity of beta hydroxybutyryl CoA dehydrogenase was 1520±222 nmoles/min/mg protein for 19 determinations on 5 air grown cultures and 1514±222 nmoles/min/mg protein for 10 determinations of 3 cultures exposed to the O_2 conditions described for thiolase. One determination of cells exposed to N_2 at 15 atm plus 1 atm of air gave a value of 1697 nmoles/min/mg protein.

The specific activity and spectrum of fatty acids synthesized was not changed by exposure of cell-free extracts of E. coli to O_2 at 15 atm, compared to air (the spectrum of fatty acids synthesized by cell-free extracts is, of course, different from that of live cells). Cell free extracts were incubated with malonyl co-enzyme, and in the presence of mercaptoethanol, hence irreversible effects on synthetase apoenzymes (not reversible SH effect) were measured. Fatty acid synthetase from baker's yeast was purified 120-fold. The enzyme showed approximately 13 bands in gel electrophoresis (this probably represents a disassociation of the component enzymes of the complex). Ultracentrifugation showed small amounts of three impurities in addition to the

enzyme complex (M.W. approximately 2.3×10^6). Electron micrographs revealed hexagonal complexes with probably six subunits similar to the published work of Lenin. The purified enzyme was exposed to oxygen at 40.2 atmospheres absolute pressure for 1 hour at 20°C. A simple assay was developed for estimating the activity of the condensation component enzyme (β-ketoacyl synthetase) of the yeast fatty acid synthetase complex. The radioactivity liberated as $^{14}CO_2$ from [1,3-^{14}C] malonyl-CoA was trapped in phenethylamine and measured by liquid scintillation spectroscopy. Three enzyme-catalyzed steps are involved: acetyl-CoA transacylase, malonyl-CoA transacylase and β-ketoacyl synthetase; however, β-ketoacyl synthetase is rate-limiting. β-ketoacyl synthetase activity was made independent of subsequent enzyme activities of the complex by excluding NADPH from the assay, thus blocking β-ketoacyl reductase and preventing fatty acid synthesis (see Section 14 for details).

Several pyridine nucleotides and derivatives were tested after it was discovered that NADH stimulated β-ketoacyl synthetase activity to a greater extent than could be accounted for by its reactivity in providing a pathway from acetoacetyl-enzyme to fatty acid synthesis. Presumably, the release of acetoacetate from the central sulphydryl of the complex is the rate-limiting step in the assay procedure. Optical assays, containing mercaptan and based on rates of oxidation of NADPH, were used to measure the activities of the complex with the following substrates: 1) acetyl-CoA and malonyl-CoA for measuring the complete reaction sequence, 2) acetoacetyl-CoA for the component steps beyond condensation through formation of butyryl-E complex, 3) acetoacetyl-CoA, plus malonyl-CoA for the complete sequence except through the initial condensation reaction, and 4) crotonyl-CoA only, for the second reduction. The condensation step was estimated separately by the radioactive assay. None of the component enzyme activities were detectably reduced by these hyperoxic exposure conditions.

Purified yeast fatty acid synthetase at 4°C was stable during 1- and 2-hr exposures to oxygen at 100 atm, but was 48% and 90% inactivated after 20 hr and 40 hr, respectively, with fatty acid synthesis measured by both radioactive and optical assays. Incubation with dithiothreitol did not restore activity. Component enzyme activities were compared before and after 40 hr in 100 atm of oxygen. Ketoacyl reductase activity was most reduced, followed by keotacyl synthetase and then acetyl transferase while malonyl transferase, enoyl reductase and palmitoyl transferase were not significantly inactivated. Special decompression techniques were developed for these high-pressure studies which resulted in only 0 to 10% inactivation of nitrogen-exposed control enzyme. In the course of the work it appeared that yeast fatty acid synthetase may contain a heretofore unreported binding site for coenzyme A. This site provides a mechanism for understanding two apparent anomalies with the current model of synthetase action: (a) how chain initiation is prevented during the chain elongation sequence, and (b) the lack of requirement for added CoA although the terminal product is known to be a CoA derivative. The CoA binding site also is predicted to participate in chain termination, a function which is not completely understood in current models. [Refer to references C, 7, 9, 16, 20, 24, 25, 35, 36.]

(5) EFFECTS OF HYPEROXIA ON INCORPORATION OF GLUCOSE AND ON BIOSYNTHESIS OF MACROMOLECULES.

The effects of hyperbaric oxygen on biosynthesis from radioactive glucose was measured. Cells were exposed and washed free of glucose by a membrane filtration procedure designed to permit exposure to 1 atm of air plus 4 atm

of oxygen or nitrogen and removal of the carbon and energy source (glucose) without altering the oxygen tension, temperature or pressure of the culture.

To measure the total [^{14}C] glucose incorporated into cells, 0.1 ml samples were removed, diluted in ice-cold MBS without glucose, filtered, washed, dissolved and the radioactivity was determined. To measure the assimilation of [^{14}C] glcuose into cellular structures, 0.1 ml samples were lysed in 0.05 ml of 1.2 M perchloric acid. The samples were kept on ice and were neutralized and diluted in 15 ml of 0.5 M tris (hydroxymethyl) aminomethane. The insoluble materials were collected by filtration using Millipore membranes (HAWP, 0.45 μm pore size). The membranes were dissolved and the radioactivity was determined.

To measure the persistence of $HClO_4$-soluble pool, cells in log phase growth in minimal basal salts (MBS) plus glucose medium were washed by centrifugation and suspended in MBS without glucose at 37°C. Cells were preincubated for 10 min and radioactive glucose was added and the cells were incubated. Prior to exposure to HPN or HPO, control samples were immediately diluted into MBS medium without glucose at 4°C, filtered through membranes (Millipore, HAWP, 0.45 μm pore size, 4 mm diameter) and washed with MBS medium at 4°C. The membranes were immersed in 1 ml of 1.9 M $HClO_4$ at 4°C and after 15 min, 0.5 ml of 1.0 M tris(hydroxymethyl)-aminomethane was added with stirring. The extracts including the membranes, were poured on to new membranes and washed three times with 0.5 ml portions of 1.0 M tris. The filtrate (1.0 ml portions) was added to 15 ml of dioxol and radioactivity was measured by liquid scintillation spectrometry. Radioactivity on membranes was also measured. Cells were then exposed to HPO and HPN (1 atm air plut 4 atm of oxygen or nitrogen, respectively) at 37°C for 30 min and decompressed. Cells were treated as described above to determine radioactivity in $HClO_4$-soluble and insoluble fractions. Starved cell controls were prepared by implanting cells on membranes which were incubated for 30 min on agar plates containing MBS medium withut glucose, which was preincubated to 37°C. Membranes containing bacteria were extracted and analysed as described above.

Exposure of Escherichia coli to oxygen at 4.2 atm produced rapid growth cessation which was readily reversed upon reincubation with air as the gas phase in minimal medium with any of three different carbon sources. General biosynthetic incorporation was also rapidly curtailed. Hyperbaric pressure, per se, did not cause these effects.

The effect of hyperoxia was further investigated by examining the rates of appearance of radioactivity in the soluble pool of the cell, and in cell structure during recovery in the presence of [^{14}C] glucose with air as the gas phase. At intervals during the early recovery period (approximately the first 2 min following oxygen exposure) the amounts of glucose which were transported, were incorporated into structures and into soluble pool, and the amount oxidized to CO_2, was determined. These data represent rates in cells during early recovery from HPO, and thus may be different from rates in cells in 4.2 atm of oxygen. In the first 5 sec, glucose was transported by both HPO- and HPN-exposed cells at comparable rates. Little synthesis of cell structure occurred but the soluble pool, which may be presumed to include glucose-6-phosphate, (the transported form of glucose) and a variety of soluble anabolic and catabolic intermediates derived from glucose, increased much more rapidly in HPN-exposed cells than in cells exposed to HPO. Since the initial transport rate was not decreased in HPO-exposed cells, it appear that the comparatively smaller soluble pool resulted, at least in part, from failure of the cells to synthesize soluble intermediates from the transported glucose.

Also, there appeared to be initially a much higher rate of production of CO_2, which would result in elimination of the transported glucose by HPO-exposed cells during recovery. [The CO_2 was measured indirectly, by difference, since no practical way was devised to measure CO_2 evolution over these time intervals]. Subsequently, transport and evolution of CO_2 in HPO-exposed cells declined and then stabilized, relative to HPN-exposed cells, while incorporation into cell structure and into the soluble pool remained low. These data strongly suggest that upon removal from HPO, cells are fully capable of transporting and metabolizing glucose to CO_2, but that the ability to synthesize soluble intermediates and more complex cell structures is severely reduced.

Indeed, when oxygen uptake was measured directly while cells were exposed to 4.2 atm of oxygen, the decline was not nearly so abrupt as the growth inhibition. This was especially true when succinate was the carbon and energy source, but even with glucose more than 50% of the normal respiration rate continued over the interval 5 to 10 min in hyperoxia. CO_2 evoluation was decreased during the interval, 5 to 20 min while the cells were in 4.2 atm of oxygen with an exogenous glucose supply available. However, it should be recalled that these comparisons were made between exponentially growing cells (air as the gas phase) and cells whose growth was practically zero (HPO as the gas phase). A comparison of CO_2 evolution from endogenous sources, where neither cells with air nor cells with HPO as the gas phase were growing, showed no significant differences. Taken, together these data do not support respiration failure as as a cause of growth inhibition in hyperbaric oxygen.

It appeared to be of interest to determine the fate of materials already present in the cell soluble pool, during oxygen-intoxication. If as suspected, biosynthesis were blocked but glucose transport were maintained, the intracellular radioactive pool should stay constant or increase during incubation in hyperbaric oxygen with radioactive glucose present in the medium. However, the pool should be depleted if transport were more rapidly and severely affected than was subsequent synthesis into cell components. As a control to partially mimic cells without transport capability but with an undamaged metabolic system, cells were incubated in the absence of glucose with air as the gas phase. As expected, the radioactivity in the intracellular pool decreased. With cells pre-labelled for 3 min, the radioactivity in the soluble pool did not decline in cells exposed to hyperbaric oxygen for 30 min. However, only 45.4×10^6 molecules of glucose, which is equivalent to 0.27×10^9 carbon atoms, were incorporated into cell structure. Approximately 1.07×10^9 molecules of glucose (or 6.42×10^9 carbon atoms) were required to construct one cell, as calculated from the data for 100% labelled cells. Thus, growth in hyperoxia was stopped after the incorporation of only 4% of the carbon required for production of a new cell; hence in less than 1/20 of a generation. With a high concentration of glucose present, rather than being depleted, the intracellular soluble pool actually increased. However, the increase was seen also in HPN-exposed cells, and was apparently pressure-related. These results also confirmed that cessation of synthesis and growth was due to failure of synthesis mechanisms, per se. Shut-down of protein synthesis was almost complete within four min. These data should not be interpreted as evidence that damage occurred specifically in translation, but that some cellular process (or processes) required for the conversion of glucose into protein, was impaired. The impaired processes do not include glucose transport or bioenergetics, but most probably involve biosynthetic enzymes. The failure of protein synthesis

could represent an early effect of oxygen toxicity and may, indeed, result indirectly from inhibition of synthesis of certain amino acids (See Section 12). [Refer to reference 37.]

(6) EFFECTS OF HYPEROXIA ON BIOSYNTHESIS OF RNA, DNA AND PROTEIN

A combination of different bacterial strains, nutritional conditions and chloramphenicol was used to suppress or allow stringency and the consequences of known sites of oxygen toxicity, and the effects of hyperbaric oxygen were measured on net synthesis by Escherichia coli of RNA, DNA, protein and lipid. With stringency mitigated by amino acids and with niacin and thiamin present the otherwise completely static effect of oxygen was abated and growth continued but with a longer generation time. The reduction of growth; and net synthesis of RNA, DNA, and protein occurred in approximately balanced amounts as determined by comparison with net synthesis by control cells with air as the gase phase. In medium without protective amino acids, induction of stringency was a principal indirect cause of inhibition of net synthesis of RNA and lipid. However, there also was evidence of significant direct impairment of RNA net synthesis. Net synthesis of DNA was reduced either because of an effect directly on DNA metabolicm or indirectly because of impaired RNA synthesis. Protein net synthesis was stopped indirectly when amino acids, whose syntheses are known to be poisoned in hyperoxia, were omitted from the medium. This occurred indirectly via the stringecy control mechanism and due to the lack of specific amino acids. However, with amino acids provided and stringency prevented there still was impaired protein synthesis in hyperoxia. This may have occurred via the observed net inhibition of RNA synthesis. Plasmolyzed cells, prepared after oxygen poisoning, showed impaired RNA and DNA net synthesis capability from nucleotides, indicating that inhibition occurred subsequent to nucleotide synthesis. These inhibitions reduce but do not prevent growth in hyperoxia. [Refer to references 39, 40, 42, 43, 50, 51.]

(7) THE EFFECT OF CARBON AND ENERGY SOURCE ON THE TOXICITY OF OXYGEN

The growth-inhibitory effect of 4.2 atm of hyperbaric oxygen for Escherichia coli is strongly influenced by available nutrients.

Glucose protected better than other intermediates later in glycolysis and in the Krebs cycle. The pattern of protection achieved in preliminary experiments with various carbohydrate intermediates was consistent with oxygen-induced poisoning of fructose-1,6-diphosphatase and of enzymes required in the pentose shunt and for converting galactose into glucose. Two of these sites were not investigated further, but preliminary evidence was obtained that purified fructose-1,6-diphosphatase was inactivated in vitro by the products generated by xanthine oxidase, but not by molecular oxygen at hyperbaric pressure (4.2 atm). Poisoning of fructose-1,6-diphosphatase would thus have deleterious effects for E. coli in media where synthesis of glucose by reverse glycolysis is required, and presumably for cells of higher organisms, including man.

The study of the effects of hyperoxia on reverse glycolysis was continued. Measurement of fructose-1,6-diphosphatase activity in extracts of oxygen-poisoned E. coli K-12 (in contrast to the effects found for enzyme exposed in vitro to hyperoxia) revealed that inactivation of the enzyme in HPO was

minimal. Further tests were done to determine potential protective effects of intermediates of glycolysis. As previously determined with strain E-26, glucose was the most protective compound, and fructose-6-phosphate was almost as good. Fructose-1,6-diphosphate and pyruvate did not protect as well. However, with tests designed to determine if the compounds were transported and utilized by cells growing as controls with air as the gas phase, only fructose-1,6-diphosphate failed to increase the total yield of cells. This clearly showed that the failure of fructose-1,6-diphosphate to protect was due to failure to taken into the cells (even in control cells) presumably because of the two highly charged phosphate groups and lack of a specific transport system. Thus, it was not due to poisoning of fructose-1,6-disphosphatase. To further establish this point, glycerol was tested and found to be a substrate. Glycerol enters the EMP prior to PEP as glyceric acid-3-phosphate, and glycerol provided protection comparable to fructose-6-phosphate. Therefore, the apparent block in reverse glycolysis clearly is not at fructose-1,6-diphosphatase but could have been due to impairment of phosphoenol-pyruvate carboxykinase and phosphoenolpyruvate synthetase.

Subsequently, direct analysis of these enzymes revealed there was no significant impairment in cells poisoned by hyperoxia of the three enzymes unique to reverse glycolysis: fructose-1,6-diphosphatase, phosphoenolpyruvate synthase, or phosphoenolpyruvate carboxykinase. Indirect evidence also suggested that protection of glucose was not due exclusively to either an elevated synthesis of superoxide dismutase, or a lesser production of toxic oxygen radicals by cells grown with glucose. Thus, the reason why glucose permits more growth in hyperoxia remains unknown. [Refer to references 40, 43, 44, 50, 51.]

(8) THE EFFECTS OF HYPEROXIA ON RESPIRATION AND ON OXIDATIVE PHOSPHORYLATION

Escherichia coli was exposed to hyperoxia from 1 to 16 atm and the oxygen uptake, measured polarographically, was compared for cells grown in air and cells exposed to hyperoxia. Exposure of membrane cultures to oxygen at 3 atm or greter produced a rapid but reversible inhibition of respiration rate. Recovery of respiration was not dependent upon growth, i.e., it occurred in the absence of a nitrogen source. When liquid cultures in exponential growth in air were exposed to oxygen at 6.2 atm, respiration was decreased from 5.67 µl/min (32 µl/min/10^{10} bacteria) to a value too low to measure (less than 0.03 µl/min). With exposure from 20 min to 2 hr, the respiration rate was totally inhibited for 5 to 7 min after removal to air; respiration then recovered at a rate proportional to the duration of hyperoxia. The rapid recovery of respiration is in agreement with previous observations that hyperoxia is primarily bacteristatic for bacteria.

Exposure to oxygen at 6 atmospheres which was sufficient to stop growth and to reduce respiration rate of Escherichia coli, did not significantly alter the oxidative phosphorylation efficiency of various cell-free fractions which when measured in air. The P/O ratio was approximately 1.00 $\pm$ 0.25 for complete extracts and 0.5 for the dialyzed fraction of greater than approximately 300.000 molecular weight. The fraction containing components of less than 300,000 in molecular weight did not phosphorylate. The rate of phosphorylation in complete extracts was sufficient to account for the production of approximately 34,400 $\pm$ 9,700 molecules of ATP from ADP/sec/bacterium. However, intact bacteria in exponential growth had approximately 59-times the oxygen uptake rate of extracts derived therefrom. [Refer to references 10, 15, 23.]

(9) EFFECTS OF HYPEROXIA ON ATP CONTENT

Escherichia coli, strain E-26, grown in defined salts medium with glucose as the sole carbon and energy source, contained $1.50 \pm 0.16 \times 10^6$ molecules of ATP/cell. ATP was extracted with $HClO_4$ and assayed with a Dupont Luminescence Biometer using the luciferin-luciferase assay.

Pressure, per se (4 atm N_2, plus 1 atm air) did not affect the growth of E. coli as measured by absorbance change at 500 nm or by incorporation of [^{14}C] glucose into cells but did result in an increase in the cellular ATP concentration. This increase was present after 5 min and was still present after 30 min of exposure but returned to normal within 15 minutes after decompression. Thus, pressurization per se did not cause a decrease in the ATP content of E. coli, but rather a slight increase. Exposure during exponential growth at 37°C to 4.2 atm of oxygen resulted in complete growth cessation within 5 min. During 2 hr of such hyperbaric oxygen exposure with ATP determinations at intervals the ATP/cell ratio fluctuated; first decreasing, then increasing, and finally decreasing again. These results would occur if the relative inhibition of ATP-yielding events, compared to inhibition of ATP-yielding events, was not constant during continued exposure. The lowest amount of ATP per cell in hyperbaric oxygen was obtained after 2 hr and was similar to the amount in energy source-deprived cells. Upon incubation with air as the gas phase, following 1 or 2 hr in hyperbaric oxygen, the ATP/cell increased slowly, compared to the increase observed after adding glucose to energy source-deprived cells.

Cultures were then incubated in hyperbaric oxygen for shorter intervals and the changes in ATP per cell during exposure and recovery were observed. Exposure to hyperbaric oxygen for a total of 30 min revealed that the ATP/cell was significantly elevated after 5 min, compared to the control, but was decreased at 30 min ($P<0.05$), and had returned to the amount found in cells prior to hyperbaric oxygen exposure within 30 min after reincubation with air as the gas phase. Thus, with briefer hyperbaric exposure, the cells restored the loss in ATP more rapidly, but not nearly so rapidly as after addition of glucose to cells with a low ATP content due to a different mechanism (energy source deprivation).

Brief exposure to hyperbaric oxygen with analyses at short intervals showed that the ATP/cell ratio, relative to control, was significantly elevated at 1, 3, and 5 min; unchanged at 10 min; and signficantly reduced at 15 min ($P<0.05$). The elevation in the ATP/cell ratio which was consistently observed during the first 10 min of hyperbaric oxygen epxosure was also seen during hyperbaric nitrogen exposure with 0.2 atm of oxygen present. This initial increase in ATP per cell with E. coli is in agreement with Britton Chance's findings (1) with mammalian cells and tissues. Chance suggested the ATP increase was due to oxygen inhibition of the pathway of reversed electron transport which can contribute significantly to ATP utilization.

Oxygen at 4.2 atm caused complete growth cessation within 5 min of exposure. A significant decrease in cellular ATP did not occur until approximately 15 min.; therefore, a decrease in cellular ATP did not cause growth cessation.

The effects of hyperbaric oxygen on the ATP concentration of cells grown in defined salts medium with glucose and with succinate were compared. The ATP/mg cells (dry weight) for the two energy sources was significantly different (at P 0.05) but they were in the same range. The effects of hyperbaric oxygen, in both cases, were a significant increase (at $P<0.05$), followed by a decrease

(1) Chance, B. Behav. Sci. 15:1-23 (1970).

in ATP/cell at 15 min. The temporary increases observed upon pressurization and upon addition of glucose to energy source-deprived cells are consistent with previously reported conclusions that ATP concentration in E. coli is not closely regulated (1).

Sanders et al. (2) reported that succinate protected rats from the toxic effect of oxygen. Rats exposed to hyperoxia showed a decrease in ATP concentration in liver, kidney and brain (3), which did not occur when the animals received succinate prior to hyperbaric oxygen exposure. The protection given these animals against the toxic effects of oxygen was postulated to be due to maintenance of the cellular concentration of ATP by succinate (3). However, we found no indication that succinate protected E. coli from the toxic effects of oxygen as measured by the effect on ATP.

The data support the following conclusions: (1) oxygen-induced growth cessation of E. coli did not occur because of a decrease in the ATP content since cell growth stopped before a measurable decline in ATP occurred; (2) exposure to 4.2 atm of oxygen resulted in a decrease in the ATP concentration within 15 min with either glucose or succinate as the sole carbon and energy source; and (3) over a 2-hr interval, hyperbaric oxygen exerted differing degrees of inhibition on ATP-producing and ATP-requiring reactions which resulted in slow, cyclic changes in the ATP/cell ratio. [Refer to references 29, 32, 37, 50, 51.]

(10) EFFECTS OF HYPERBARIC OXYGEN ON TRANSPORT

The analysis of potential direct effects of oxygen poisoning on transport in Escherichia coli is complicated by several factors. Poisoning of metabolic utilization of the substrate being transported would result in subsequent, but indirect, reduction of uptake which could be interpreted as impaired transport. Initial transport rates should be measured, rather than transport rates measured over time periods when transport, even in controls, may not be maximal. Such time periods were unknown, but there was no assurance that initial, maximal rates lasted for more than seconds. Bacteria poisoned by hyperbaric oxygen were observed to recovery rapidly with respect to many parameters, with recovery beginning within seconds to minutes after decompression. Thus, potential transport effects had to be studied quickly, and preferably with as little manipulation of the cells as possible.

Preliminary findings suggested that transport of glucose and of acetate was decreased by exposure of E. coli to hyperbaric oxygen. Correlated studies of respiration, uptake, assimilation, CO_2 evolution, ATP synthesis from glucose, incorporation into soluble and into TCA-insoluble fractions, the use of non-metabolizable substrates, and the development of an analytical instrument to permit rapid analysis of transport all contributed to solutions of these difficulties. Transport was measured in samples removed from the hyperbaric chamber by an instrument which automatically added radioactive glucose to a stirred cell suspension at 37°C, and filtered and stored 10 samples over 10 seconds. HPO-exposed cells initially transported glucose at a rate similar to HPN-exposed cells. However, after a few seconds the transport rate of the HPO-exposed cells markedly decreased while the control cells main-

(1) Cole, H.A., Wimpenny, J.W. and Hughes, D.E. Biochim. Biophys. Acta 143: 445-53 (1967).

(2) Sanders, A.P., Hall, I.H. and Woodhall, B. Science 167:1508-10 (1970).

tained a higher transport rate. However, control cells continued to grow exponentially, while the growth of HPO-exposed cells, was inhibited.

Capability to transport thiomethyl galactose (TMG) was maintained by intact cells which were tested after removal following exposure for 1 hr to 4.2 atmospheres of oxygen. The rates were not significantly different ($P<0.05$) compared to control cells similarly exposed to HPN for 5 min or 10 min. Similar results were obtained when TMG was added without decompressing the cells and rates were measured over the interval 5 min to 10 min.

Experiments were designed to compare rates of facilitated transport by membrane vesicles [Kabackasomes (1)] following exposure to air, and to mixtures of 1 atm of air plus 4 atm of either nitrogen or oxygen. Membrane vesicles are osmoregulating structures devoid of cell wall and internal enzymes. Such vesicles are capable of transporting (but not metabolizing) glucose by the phosphoenol-pyruvate phosphotransferase system. The differences in transport of glucose by vesicles exposed to air and by vesicles after exposure for 1 hr at 4.2 atm of oxygen or 1 atm of air plus 4 atm of nitrogen were not significant at $P<0.05$.

Similar experiments were done, except with exposures for 1 hr to 50 atm of oxygen or nitrogen and 1 atm of air. Again, the results showed no inhibition by hyperoxia. Specifically, the glucose transported during 1, 2, 5 and 10 min revealed no significant differences ($P<0.05$) between 50 atm of oxygen and 50 atm of nitrogen or 1 atm of air, except for one of the twelve assay intervals (2 min in HPO was lower than 2 min in HPN). Subsequently such membrane vesicles were found to be unaffected by exposure for 2 hrs at 50 atm of oxygen.

Respiration of glucose and succinate in intact cells was measured polarographically by a technique which permitted rate measurements under hyperbaric oxygen. Growth completely stopped during the first 3 min, but 88% and 66% of the normal respiration for succinate and glucose, respectively, remained over the interval 5 to 10 min at 4.2 atm of oxygen. ATP concentration was significantly increased during the first 10 min at 4.2 atm of oxygen and declined only after growth was stopped. Cells in exponential growth in air contained $(1.54 \pm 0.12) \times 10^6$ molecules of ATP/cell. After washing free of glucose, the ATP content of the starved cells was decreased to about 30% of normal. Oxygen-exposed and controls cells showed similar capabilities to accumulate ATP in the first few seconds after glucose was resupplied, verifying that glucose is transported and that a pathway (probably via the cytochromes) to production of ATP is functional.

Radioactive glucose accumulated in the soluble pool during hyperbaric oxygen exposure, but incorporation into cell structure stopped abruptly. Under these conditions, during 30 min in 4.2 atm of oxygen, cells incorporated fewer molecules of glucose into cell structure than did control cells in air in 3 min (45.4×10^6 vs. 57.2×10^6 glucose molecules per cell, respectively). However, radioactivity from glucose in the medium continued to accumulate in the intracellular pool of soluble metabolites, and the amount increased from 57.2×10^6 to 70.6×10^6 equivalent molecules of glucose per cell during hyperbaric exposure while control cells which were starved of glucose in air, showed a steady decline in intracellular soluble pool (these cells were used as a control for

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(1) Kaback, H.R. Methods in Enzymology 22:99-120 (1971).

cells incapable of transport, but with no damage to biosynthetic machinery). These data verified that transport was functional but that incorporation was seriously impaired in hyperbaric oxygen. [Refer to references 23, 26, 27, 37.]

(11) EFFECT OF HYPEROXIA ON THIAMIN METABOLISM

Thiamin, as well as hydroxymethylpyrimidine (HMP) and methylthiazole (MT) (two intermediates in separate pathways leading to moieties which are condensed to form the vitamin) is beneficial for growth of E. coli exposed to toxic concentrations of oxygen. The addition of the intermediates HMP and MT to culture medium was used to search for a possible enzymatic inhibition site in thiamin biosynthesis. The data were inconclusive, since both HMP and MT provided benefit alone or together. These results (stimulation by either alone) are not compatible with a simple impairment of biosynthesis since two branch pathways are involved. It is conceivable that the added intermediates may each stimulate the opposite arm of the pathway, resulting in restoration of synthesis even in the face of damage to an enzyme in the pathway.

To gain further insight, the concentration of thiamin has been measured in oxygen-poisoned E. coli. Both fluorometric and microbiological assays show that thiamin is reduced in concentration by more than 90% after 4 hrs in 4.2 atm of oxygen. After only 30 min, thiamine is reduced by more than 50% and this reduction is almost entirely prevented by inclusion of HMP in the medium.

If thiamin biosynthesis is impaired, the mechanism does not have relevance to human oxygen toxicity since humans lack this biosynthetic pathway. If, however, hyperbaric oxygen leads to chemical destruction (oxidation) of thiamin, there is potential significance for human toxicity due to hyperbaric oxygen, and to redox-active chemicals. [Refer to references 34, 38, 40, 42, 43, 50, 51.]

(12) INHIBITION OF AMINO ACID BIOSYNTHESIS AND INDUCTION OF STRINGENCY

To further study the biosynthetic inhibition, nutritional supplementation was used. Enrichment of media with yeast extract protected against oxygen toxicity. This protection resulted because of the presence of the following amino acids: valine, tyrosine, isoleucine, tryptophane, leucine, phenylalanine, cysteine, methionine, asparagine, and threonine (listed in order of significance for protection). These data, plus information gained through the use of intermediates in the biosynthetic pathways, suggest that specific enzymes in biosynthesis of these amino acids (particularly one enzyme each in the aromatic and branched-chain pathways) are extremely sensitive to oxygen, being totally inactivated within less than 5 min with 4.2 atm of oxygen in the gas phase. The inability to synthesize these amino acids (and several vitamins and cofactors via branches from amino acid biosynthetic pathways) is sufficient to account for the initial growth cessation which occurs when E. coli is placed in hyperbaric oxygen in minimal medium.

We theorized that inhibition of biosynthesis of specific amino acids would lead to induction of stringency since amino acid starvation is the trigger for

ppGpp induction. Within 2 min after HPO exposure, ppGpp began to increase in stringent but not in relaxed strains, reaching 1.2×10^6 molecules/cell in 10 min. We also determined that strains, isogenic except for the stringecy gene, differ in their resistance to oxygen poisoning. The stringent strain, like most wild strains, survived 4.2 atm of oxygen with little or no killing for 21 hrs, while the relaxed strain showed a 35% decrease in viability. Inhibition of growth was reversed in the stringent strain within 3 hr, but requried 16 hr in the relaxed strain. Therefore, stringency contributes to the mechanism of growth inhibition and provides a biologically significant protective role by shutting down the cell biosynthesis in concert and preventing detrimental, unbalanced growth.

Experiments on the specific site of inhibition of the biosynthesis of branched-chain amino acids by hyperoxia were also concluded. Dihydroxy-acid dehydratase was approximately 78% reduced in specific activity in extracts prepared from E. coli cells exposed to 4.2 atm of oxygen for only 10 min. Aceto-lactate synthetase specific activity was not significantly altered under such conditions. There was a small (20%) decrease of doubtful biological significance in dihydroxyisovalerate dehydrogenase specific activity. Unless branched-chain amino acids are supplied, protein synthesis and growth stops abruptly. The sensitivity of dihydroxyacid dehydratase thus accounts for the observed necessity of having the branched-chain amino acids in the culture medium since they cannot be synthesized adequately during HPO exposure. [Refer to references 31, 34, 39, 40, 42, 43, 45, 46, 47, 49, 50, 51.]

(13) INHIBITION OF PYRIDINE NUCLEOTIDE BIOSYNTHESIS BY HYPERBARIC OXYGEN

The concentrations of the pyridine nucleotide coenzymes in E. coli were measured to determine if changes in the intracellular coenzyme pools were associated with the toxic effects of hyperoxia. Oxygen at 1 atm increased the generation time by 33% compared to air and NAD was decreased by 30%. Oxygen at 6 atm was immediately bacteristatic and 2 hours of exposure produced a 62% decrease in NAD. NADPH was present in normal concentrations in oxygen-inhibited bacteria and should not be limiting for reductive steps in synthesis, including the synthesis of fatty acids.

The decreased coenzyme concentrations were thought to have resulted from inhibition of coenzyme biosynthesis. Two enzymes were possible sites of inhibition: phosphoribosylpyrophosphate (PRPP) synthetase and quinolinate transferase. Quinolinate transferase was suspected, because niacin and intermediates beyond quinolinate, but not quinolinate, protected cells from hyperoxia. PRPP is also required for this reaction. Therefore, direct tests of the activities of both enzymes were done on control cultures grown in air ad on cells following exposure to growth-inhibiting concentrations of oxygen.

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, since man also requires this enzyme for de novo coenzyme biosynthesis.

NAD and NADH were shown to be severely decreased in cells during poisoning by hyperoxia. The NAD decrease in 5 min was statistically significant at $p<0.05$ and had decreased from $918\pm161\times10^3$ molecules/cell in air-growth control cells to $58\pm57\times10^3$ molecules/cell in cells exposed for 2 hrs to 4.2 atm of oxygen. This decrease was prevented by including niacin, but not quinolinate in the medium which is consistent with poisoning of quinolinate phosphoribosyltransferase as the site of poisoning.

NADP and NADPH were present in much lower concentrations in control cells and changed less during oxygen poisoning. The total of the 4 forms of the coenzymes (NAD, NADH, NADP and NADPH) decreased from $1726\pm257\times10^3$ molecules/cell to $260\pm27\times10^3$ molecules/cell in 2 hrs at 4.2 atm of oxygen. Concurrently the generation time increased from 26.8 ± 0.9 to 134 min. The medium contained 20 amino acids to prevent known growth inhibition via impaired biosynthesis of amino acids. The oxidized to reduced ratios of the conezymes were not maintained during oxygen poisoning but decreased from 1.43 ± 0.09 to 0.44 ± 0.18 for NAD/NADH, and from 0.70 ± 0.26 to 0.49 ± 0.15 for NADP/NADPH during 2 hrs at 4.2 atm of oxygen.

Finally, 5-phosphoribosyl pyrophosphate (PRPP) does not protect against oxygen poisoning and PRPP synthetase is not affected by hyperoxia, but PRPP does accumulate in oxygen poisoned cells. Accumulation of PRPP is in agreement with inhibition by hyperoxia of quinolinate phosphoribosyltransferase, which uses PRPP as a substrate. PRPP was also shown to accumulate in human red blood cells exposed to hyperoxia. [Refer to references 8, 13, 38, 40, 42, 43, 48, 50, 51.]

(14) SPECIALIZED INSTRUMENTATION AND ANALYTICAL TECHNIQUES DEVELOPED DURING THE RESEARCH

Instrumentation

(1) Programmable Instrument for Automated Determination of Transport Rates.

An automated sampling electronic instrument was designed to permit determination of rates of biological transport of metabolites into cells. The substrate was automatically introduced into a stirred cell suspension at 37°C. The first sample was automatically taken at programmable intervals (1 to 100 sec). The samples were forced by pressure differential (vacuum) through 0.4 μ pore size membranes and approximately 50 μl were collected in disposable cups. The duration of the sampling interval was controllable down to 0.1 sec. The samples preserved records of the substrate concentrations in solution at the times of filtration. With the use of suitable radioactive labelled isotopes, the changes in substrate concentrations may be conveniently measured by liquid scintillation spectrometry, but other analytical procedures of suitable sensitivity may be used. Initial and steady-state transport rates of succinate and glucose in Escherichia coli were obtained using the device.

(2) Pressurized Fermentation Vessel

Microorganisms can be used advantageously to study the effects of various gaseous environments at elevated pressures. For enzymatic and other biochemical studies several grams of biomass may be the minimum from which meaningful data can be obtained. A fermentor is a convenient means of obtaining bacteria

in these quantities. A chamber was constructed to enclose the fermentation and thus convert it to pressure fermentation. It was applied in studies of oxygen toxicity at pressures up to 6 atmospheres. Temperature was maintained by the thermoregulating system of the fermentor and temperature monitoring was continuously available. Samples were routinely obtained during fermentation at 6 atmospheres pressure with flow rates up to 120 ml min through the sample port.

Since this chamber was designed for use with oxygen it was painted with a special inorganic paint (Polythane, Preserv-O-Paint Company, Houston, Texas) prepared to the specifications of the National Aeronautics and Space Administration. The chamber was tested before use by filling it partially with water and pressurizing with compressed air. For this test the safety valve was replaced with a brass plug. In the pressure test, the chamber began to leak around the O-ring at 500 lbs per sq. in., which was 4 times the design operational limit. The O-rings are the weakest point in the system and serve as a safety feature in addition to the pressure release valve.

Analytical Procedures

(1) Simple assay for the condensation component enzyme (β-ketoacyl synthetase) of fatty acid synthetase.

A simple assay was developed for estimating the activity of the condensation enzyme (β-ketoacyl synthetase) of the yeast fatty acid synthetase complex. The radioactivity liberated as $^{14}CO_2$ from [1,3-^{14}C] malonyl-CoA was trapped in phenethylamine and measured by liquid scintillation spectrscopy. Three enzyme-catalysed steps are involved: acetyl-CoA transacylase, malonyl-CoA transacylase and β-ketoacyl synthetase; however, β-ketoacyl synthetase is rate-limiting. β-Ketoacyl synthetase acitivity was made independent of subsequent enzyme activities of the complex by excluding NADPH from the assay, thus blocking β-ketoacyl reductase and preventing fatty acid synthesis. By this assay β-ketoacyl synthetase activity was about 0.28 of the activity of the complex for fatty acid synthesis, compared with approximately 0.001 for published assays. Several pyridine nucleotides and derivatives were tested after it was discovered that NADH stimulated β-ketoacyl synthetase activity to a greater extent that could be accounted for by its reactivity in providing a pathway from acetoacetyl-enzyme to fatty acid synthesis. Presumably, the release of acetoacetate from the central sulphydryl of the complex is the rate-limiting step in the assay procedure. [Refer to references 13, 27, 35.]

(III) CONCLUSIONS

Based on the experimental data, niacin and thiamin show promise and should be evaluated for protective and therapeutic effects against the toxicity of elevated oxygen tensions with the objectives of safely extending man's capabilities to breathe oxygen: (a) in the SCUBA rebreather, (b) during decompression, and (c) for the treatment of aeromebolism, anaerobic infections, and cardiovascular-pulmonary disorders. The biochemical evidence indicates that the cellular requirements for these vitamins increase when "oxidant-stress" is elevated and they may lessen the toxicity of various redox-active chemcals

such as the herbicide paraquat, the cancer chemotherapeutics doxorubicin and mitomycin-C, the antibiotic nitrofurantoin, and environmental pollutants such as hydrazine, ozone and other oxidants.

(IV) MAJOR ACCOMPLISHMENTS

The major accomplishments were the attainment of a broad understaing at the subcellular level of the mechanisms by which oxygen poisons E. coli and circumvention of this damage. These solutions potentially have significant applications to higher systems, presumably to man, and to chemicals which (through redox-cycling) share mechanisms of toxicity with oxygen.

Specific accomplishments are listed as follows:

1. Hyperbaric oxygen is static, not cidal for Escherichia coli.

2. Carbon dioxide deprivation kills E. coli in pure oxygen environments when low population densities can not supply required carbon dioxide.

3. The fatty acid synthesis machinery of E. coli and bakers' yeast, studied in vivo and in vitro, is very resistant to hyperoxia, even though it is dependent on SH groups for function.

4. Deficiency of ATP is not the cause of growth inhibition of E. coli by hyperoxia.

5. Direct inhibition of the biosynthesis of the macromolecules, DNA, RNA, protein and of lipids, as well as impairment of oxidative phosphorylation or transport is not the cause of growth inhibition of E. coli by hyperoxia.

6. Poisoning of specific enzymes in several amino acid biosynthetic pathways is an early event in oxygen poisoning of E. coli.

7. Poisoning of amino acid biosynthesis induces stringency, which indirectly impairs RNA synthesis and many other biosynthetic processes, leading to rapid, reversible growth inhibition in medium deficient in amino acids.

8. When amino acids were supplied, a requirement for niacin and for thiamin was demonstrated in E. coli poisoned by hyperoxia. Whether thiamin biosynthesis was impaired or whether thiamin was destroyed was not proven. Niacin is beneficial because quinolinate phosphoribosyl transferase, an enzyme required for its de novo biosynthesis, is poisoned.

9. Poisoning of quinolinate phosphoribosyl transferase leads to rapid decreases in the pyridine nucleotide coenzyme concentrations which limit growth in the absence of added niacin.

10. Niacin protects bacteria and theoretically should protect man against oxygen-mediated poisoning of NAD biosynthesis.

11. Hyperoxia impairs DNA and RNA synthesis by a direct means in addition to stringency induction, but growth at a reduced rate continues.

12. There is preliminary evidence that oxygen poisons an enzyme required for conversion of galactose into glucose, suggesting that galactose accumulation would occur in tissues of higher animals poisoned by hyperoxia.

13. Glucose was found to be superior to any other intermediate of the Krebs cycle or of glycolysis as a carbon and energy source for E. coli exposed to hyperoxia, for reasons which were not determined; however, it was not due to poisoning of an enzyme unique to reverse glycolysis, a possibility otherwise compatible with the observations.

14. In work beyond the contract support, several of these findings have now been extended to higher systems, theoretically including man, and the explanations of hyperbaric oxygen toxicity were successfully applied as partial explanations for paraquat toxicity, and for the toxicities of nitrofurantoin and doxorubicin, and perhaps to other redox-active compounds which have propensities to form free radicals, both from the compounds and from oxygen in aerobic tissues and cells.

(V) INDEX OF TECHNICIANS AND STUDIENTS WHO RECEIVED TRAINING AND SUPPORT

Twenty-four people received training and/or educational support during the course of this contract, including 5 at the B.S. Level, 6 at the M.S. Level, 2 at the doctoral level, and 2 at the postdoctoral level. Two other student programs at the masters and doctoral level derived indirect benefits. Subsequently four individuals have gone on to Ph.D., M.D. or D.D.S. degrees elsewhere. Following is a list of those supported by the contract.

H.F. Howitt, B.S. to M.S., 1969-71; S. Major, B.S., 1969-71; J.L. Stees, B.S. To M.S. to Ph.D., 1969-73; R. Davis , undergraduate, 1969; R. Brunker, B.S. to M.S., 1970-72; W. Yang, B.S., 1971; C. Cunningham, B.A., 1971-72; S. Weaver, M.S., 1972-73; D. Peterson, Undergraduate 1972-73; Margaret Lu , B.S., 1973; Fred Yein, M.S., 1974-79; Mike Ballady, B.S., 1974; Rebecca Mathis, B.A. to M.S., 1974-75; Steve Cohle, B.A., 1974; Mike Hines, Undergraduate to B.A., 1974; Kim Vincent, B.A., 1975-76; Laura Leung, B.S., 1975; Daniel Boehme, M.S. 1976-77; Richard Seither, B.S. to M.S. (currently working toward Ph.D.), 1978-81; Laurie Foudin, Ph.D., 1979-80; Patti Gilliland, M.S., 1979-80; Doris Song, M.S., 1979-81; Charles Fisher, Ph.D., 1980; and Daisy Perez, M.S., 1981.

(VI) INDEX OF REPORTS SUBMITTED TO OFFICE OF NAVAL RESEARCH

1.	First Six Months Report.	June 10, 1968
2.	Request for Continuation for 1969.	Sept. 6, 1968
3.	Progress Report Abstract (DR-146 pp. 50-51, 1969).	Dec. 12, 1968
4.	Request for Continuation for 1970.	July 10, 1969
5.	Progress Report Abstract (no DR #, pp. 49-50)	Nov. 19, 1969
6.	Supplementary Report: Abstracts of papers submitted	Dec. 2, 1969
7.	Supplementary Progress Report.	April 7, 1970
8.	Request for Continuation for 1971.	July 17, 1970
9.	Progress Report Abstract (ACR-174, pp. 49-50	Dec., 1970
10.	Technical Report No. 1	Jan., 1971
11.	Technical Report No. 2	April, 1971
12.	Request for Continuation for 1972.	July 13, 1971
13.	Progress Report Abstract (ACR-180, pp. 19-20)	Dec., 1971
14.	Technical Report No. 3 (annual report for 1971).	Jan., 1972
15.	Six months progress (status) report and Request for Continuation for 1973	June 1, 1972
16.	Technical Report No. 4	July, 1972
17.	Progress Report Abstract (ACR-189, pp. 93-94).	Dec., 1972
18.	Technical Report No. 5 (Annual Report for 1972).	Jan. 1973
19.	Six months progress (status report and request for continuation)	June, 1973
20.	Progress report abstract (ACR-197, pp. 39-40).	Dec., 1973
21.	Technical report No. 5 (Annual Report for 1973).	Jan., 1974
22.	Six months progress (status) report and request for continuation	June, 1974
23.	Progress Report abstract (ACR-2, pp29-30).	Dec., 1974
24.	Annual Report for 1974.	Jan., 1975
25.	Six months progress (status) report and request for continuation.	June, 1975
26.	Progress report abstract (ACR-219, pp. 27-28).	Dec., 1975
27.	Annual Report for 1976..	Jan., 1976
28.	Annual Report and request for continuation	Jan., 1977
29.	Progress report Abstract (ACR-225, pp. 27-28).	May, 1977
30.	Status report, and request for continuation)	Dec. 29, 1977
31.	Progress report abstract (ACR-227, pp. 101-102).	Dec., 1977
32.	Status report (Annual Report)	Jan. 1, 1978
33.	Progress report Abstract (ACR No. pp. 95-96)	Dec., 1978
34.	Six month status report and request for continuation	Dec. 31, 1979
35.	Progress report Abstract for 1980	Dec., 1979
36.	Annual Report (#4)	July 30, 1980
37.	Six month status report and request for continuation	May, 1981
38.	Progress Report Abstract for 1981.	May, 1981
39.	Final Report	June, 1982

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*Paper 33 was not directly a part of the contract work but is included because of its relevance.

**Papers 52-57 concern the mechanisms of toxicity of paraquat and adriamycin which were not directly a part of the contract, but were based on knowledge from the contract research ad supported by other funding.

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Studies using vitamins, precursors, and intermediates with analysis of carbohydrate, amino acid, lipid, protein, RNA and DNA synthesis and of enzyme activities in suspected pathways support the following events during oxygen intoxication of Escherichia coli. Biosynthesis of several amino acids are inhibited. Within less than 5 minutes valine is limiting for protein synthesis (subsequently 9 other amino acids become limiting). The resultant unloaded

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tRNA's stimulate the "stringent response" (production of the powerful inhibitor guanosine tetraphosphate) which completely stops biosynthesis.

Amino acids protect from the stringent response but quinolinate phosphoribosyl transferase, an enzyme required for de novo synthesis of NAD and NADP, also is oxygen-sensitive and coenzyme concentration falls. Niacin, which enters this biosynthetic pathway below the poisoned enzyme, prevents the inhibition of coenzyme biosynthesis and reduces the growth inhibition. Thiamin further reduces the growth-inhibition and thiamin concentration is rapidly reduced either by destruction or blockage of synthesis. With stringency mitigated and optimal nutritional protection, net synthesis of RNA, DNA, and protein was decreased but occurred in balanced amounts. In medium without Plasmolyzed cells, prepared after oxygen poisoning, showed impaired RNA and DNA net synthesis from nucleotides.

The enzymes of glycolysis, Krebs cycle and oxidative phosphorylation, and the transport machinery appear to be relatively resistant and remain functional for at least 4 generations. ATP deficiency is not the cause of growth inhibition, neither is growth inhibition caused by an overall change in oxidation state of the cellular SH groups.

Some of these sites (including quinolinate phosphoribosyl transferase, required for NAD synthesis) should be relevant to man. The data suggest that the vitamins thiamin pyrophosphate and niacin should be tested as potential protective agents against the toxicity in man of oxygen and of chemicals which produce cellular oxidative stress.

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