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ATP CONCENTRATION IN *ESCHERICHIA COLI* DURING OXYGEN TOXICITY

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SUMMARY

Escherichia coli, strain E-26, grown in defined salts medium with glucose as the sole carbon and energy source, contained $1.50 \pm 0.16 \cdot 10^6$ molecules of ATP/cell. ATP was extracted with $HClO_4$ and assayed with a Dupont Luminescence Biometer using the luciferin-luciferase assay. Exposure during exponential growth at 37 °C to 4.2 atm of oxygen resulted in complete growth cessation within 5 min, and to cyclic changes in cellular ATP concentration over a 2 h period. However, significant decrease in cellular ATP concentration occurred after growth inhibition in hyperbaric oxygen; hence, lack of ATP was not the cause of growth inhibition from oxygen toxicity.

INTRODUCTION

Oxygen is required for aerobic life but concentrations above that of air become toxic and may be lethal (for a review, see Haugaard [1]). The toxicity in mammals includes acute central nervous system effects (convulsions) and chronic lung damage. Oxygen toxicity produces cidal and static effects and other evidence of toxicity in microorganisms (for a review, see Gottlieb [2]). In *Escherichia coli*, growth and respiration was reversibly inhibited by hyperbaric oxygen [3]. There was no detectable damage with respect to the rate of oxidative phosphorylation [4]; however, there was a decrease in NAD concentration in *E. coli* exposed to hyperbaric oxygen [5]. CO_2 deprivation was responsible for a lethal effect of pure oxygen on this microbe [6].

The mechanism of oxygen toxicity at the cellular level has been the subject of considerable research. Reviews of this subject may be found in the previously cited articles [1, 2]. Controversy exists concerning whether cellular ATP is reduced by exposure to toxic concentrations of oxygen. Several research groups have published data showing a decrease in ATP in tissues of convulsed animals [7–10]. Chance et al. [11, 12] and Allen et al. [13] using different species, found that hyperbaric oxygen

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produced an increase in ATP and in the NAD/NADH ratio, although they interpreted these findings differently. Recently, Faiman and Nolan [14] reported no change in ATP in tissues of animals which were exposed to hyperbaric oxygen, then sacrificed and frozen in liquid nitrogen before decompression.

ATP concentration in relation to the onset of oxygen toxicity in single-celled systems has not been reported, to the authors' knowledge. The research in this paper was done to determine potential correlations between cellular ATP concentration and the know inhibition of growth and respiration [3] in *E. coli* exposed to hyperbaric oxygen.

MATERIALS AND METHODS

E. coli, strain E-26 [5], was maintained in a defined salts medium [5] with a single carbon source of glucose at a final concentration of 0.028 M. Where indicated, succinate (0.028 M) was substituted for glucose. Cells per ml, absorbance of cultures, and dry weight were determined, respectively, by standard plate colony counts, by spectrophotometric measurements at 500 nm wavelength, and by weighing bacteria that were implanted, washed, and dried to constant weight on 47-mm diameter membranes with a pore size of 0.4 μm (Nuclepore Corp., Pleasonton, Calif.).

Bacteria were exposed to hyperbaric oxygen as follows. Exponentially growing cultures (150 or 50 ml, respectively) were transferred either to Model 202 or Model 52 pressure vessels (Amicon Corp., Lexington, Mass.) with dilution to an absorbance of 0.5. The vessel was modified with a valve through which samples could be removed without decompressing the culture. After an interval of growth, the vessel was pressurized with 4 atm of either medical grade oxygen or nitrogen plus the 1 atm of air which was included to provide necessary CO_2 [6]. Samples were removed periodically and ATP was immediately extracted as described below. Samples (0.5 ml) were also pipetted into test tubes on ice for determination of absorbance. In some experiments, the vessel was fitted with a probe from a Beckman Model 777 oxygen analyzer which was modified to measure oxygen concentrations up to 3400 Torr.

Bacterial ATP was extracted with $HClO_4$ in procedures similar to those previously described [15]. ATP was also extracted, where indicated, as follows: 0.1 ml of culture was added to 0.9 ml of 90 % dimethylsulfoxide in 0.01 M morpholinopropane sulfonic acid buffer, adjusted to pH 7.4 with NaOH, and the tube was immediately mixed on a Vortex mixer. (It is essential that culture samples be added directly into the extraction fluid without delay.) After 2 min at room temperature, 5.0 ml of 0.01 M morpholinopropane sulfonic acid buffer, pH 7.4, was added and the solution was placed on ice until assayed, within 15 min. Variations of this procedure were tested: the length of the time between addition of the culture to 90 % dimethylsulfoxide and addition of the morpholinopropane sulfonic acid buffer was varied from 0.5 to 3.0 min; the entire extraction was conducted at 4 °C; and 1.2 M $HClO_4$ was incorporated into the 90 % dimethylsulfoxide, and neutralized after 2 min with 0.72 M KOH containing 0.12 M $KHCO_3$. Samples of the culture medium also were filtered through 0.45 μm pore size cellular acetate membranes (Millipore Corp., Bedford, Mass.) to remove bacteria. The filtrate was extracted, ATP was assayed as described below, and the amount was subtracted from ATP of the culture.

ATP was assayed using a Dupont Luminescence Biometer (E. I. Du Pont

De Nemours and Co., Inc., Wilmington, Del.). Because of the sensitivity of the method, low light response water and chemically clean glassware were used. Low light response water was prepared by boiling deionized water containing 1 drop per l of 10 M HCl, neutralizing with NaOH and autoclaving. Chemically clean glassware was prepared by rinsing clean glassware three times with 2 M HCl and three times with low light response water and air drying. 90 % dimethylsulfoxide was prepared in low light response water containing 0.01 M morpholinopropane sulfonic acid buffer, pH 7.4. ATP extract or standard (0.01 ml) was injected into the assay reagents by a spring-loaded Hamilton Reprojector 50 μl syringe. In order to prevent sample carryover between injections, the syringe was rinsed three times with 2 M HCl and nine times with 0.01 M morpholinopropane sulfonic acid buffer, pH 7.4, between each sample.

The Biometer was standardized each day with a solution of $1 \cdot 10^{-4}$ g/l of ATP which was prepared in 0.01 M morpholinopropane sulfonic acid buffer, pH 7.4. The ATP concentration was confirmed by measuring the absorption at 259 nm (molar extinction coefficient of ATP at 259 nm, $1.540 \cdot 10^4$). The ATP standard was frozen in 0.5-ml quantities and stored. Standards were used within 10 min after thawing.

Energy source-deprived bacteria were prepared by placing 5 ml of an exponentially growing culture at an absorbance of approx. 1.5 into a centrifuge tube containing 25 ml of defined salts medium [5] minus the carbon source at 4 °C. The suspension was centrifuged at 4 °C and resuspended three times in fresh medium. 3 ml of the suspension (absorbance 0.5–0.6) was incubated at 37 °C with stirring. Samples were taken periodically before and after addition of glucose to a final concentration of $2.7 \cdot 10^{-2}$ M. ATP was extracted with $HClO_4$ and measured as previously described.

Statistical probabilities were determined using Student's *t*-test.

RESULTS

Comparisons of several extraction conditions for ATP are shown in Table I.

TABLE I

COMPARISON OF EXTRACTION CONDITIONS FOR *E. COLI*

Details of extraction and assay methods are given in Materials and Methods. The mean ±S.D. for three determinations on each of two extractions are shown. The results are expressed as molecules × 10^{-6} ATP/cell.

Extraction time (min)	23 °C		4 °C	
	Neutral dimethylsulfoxide[a]	Acidic dimethylsulfoxide[b]	Neutral dimethylsulfoxide[a]	$HClO_4$
0.5	1.36±0.06	1.01±0.02	1.68±0.15	–
1	2.77±0.66	0.89±0.08	1.45±0.09	–
2	1.67±0.34	1.14±0.08	1.46±0.11	–
3	1.45±0.06	1.25±0.17	1.37±0.13	–
15	–	–	–	1.49±0.11

[a] 90 %.
[b] 90 % dimethylsulfoxide containing 1.2 M $HClO_4$.

The $HClO_4$ procedure which has been recommended previously [15] and dimethylsulfoxide for 3 min at 23 °C gave comparable results; however, dimethylsulfoxide extraction is simpler.

The generation time of cells grown with glucose as the sole carbon source was approx. 40 min. Growth was halted within 5 min by exposure to hyperoxia, but not by pressure, per se, as was demonstrated by measurements of the absorbance of the culture (Fig. 1) and by incorporation of [^{14}C]glucose (Fig. 2). Upon decompression, exponential growth returned, indicating the effect was bacteristatic. When appropriate comparable dilutions of the culture were plated immediately prior to pressurization and after 60 min with a gas phase of 4.2 atm of oxygen, there was no change in plate colony counts (125 ± 9.5 vs. 124 ± 19, average$\pm$S.D. for four plates pre- and post-oxygen, respectively). Microscopic examination after exposure of *E. coli* to 4.2 atm of oxygen with glucose as the carbon source revealed small spherical cells which returned to short rod shapes during recovery with air as the gas phase.

The relationships among colony-forming units/ml, absorbance, and dry weight/cell and the concentration of ATP in cells grown with air as the gas phase and with glucose or succinate as the sole carbon source are shown in Table II.

The ATP concentrations in cells metabolizing glucose as the sole carbon and energy source during hyperbaric intervals of 120, 60, 30 and 15 min, and following recovery after these intervals of exposure, are shown in Table III–V, respectively.

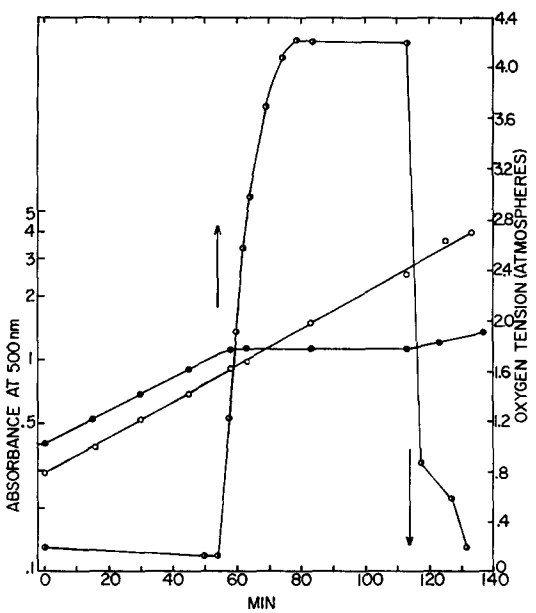

Fig. 1. Reversible inhibition of growth of *E. coli* by exposure to hyperbaric oxygen but not by pressure, per se. Cultures were grown with air as the gas phase, in defined salts medium [5] with glucose as the sole carbon and energy source, and exposed to a mixture of 1 atm air plus 4 atm pure oxygen (● — ●), or to a mixture of 1 atm air plus 4 atm nitrogen (○ — ○). Oxygen concentration in the culture exposed to hyperbaric oxygen was measured polarographically (◑ — ◑). Cultures were pressurized at the point of the upward arrow and decompressed at the point of the downward arrow. An absorbance of $1.0 = 3.07 \cdot 10^8$ c.f.u./ml, and the generation time prior to hyperbaric exposure was 37 min.

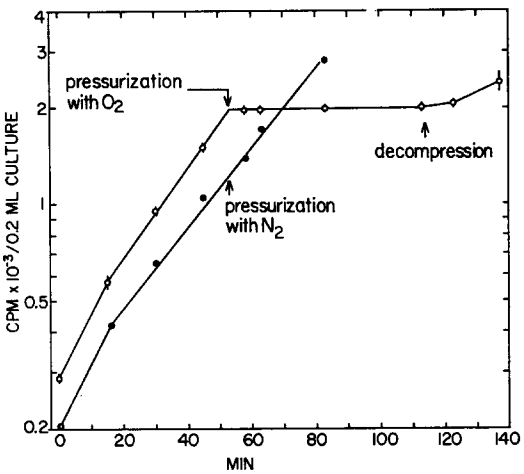

Fig. 2. Inhibition of incorporation of glucose by *E. coli* upon exposure to hyperbaric oxygen. Cells were incubated at 37 °C in defined salts medium with glucose as the sole carbon source ([U-^{14}C]-glucose; 27.7 mM, 0.506 · 10^{-2} Ci/mol). At 53 min one culture was pressurized with 4 atm of oxygen plus 1 atm of air (○—○) and decompressed at 113 min. At 53 min one culture was pressurized with 4 atm of nitrogen plus 1 atm of air (●—●). At intervals, 1.5-ml samples were withdrawn (without decompressing the parent culture) into ice-cold test tubes, and triplicate 0.2-ml samples were diluted in 15 ml of ice-cold defined salts medium without glucose contained in microanalysis filter holders with type HA, 0.45 μm pore size membranes (Millipore Corp., Bedford, Mass.). The samples were vacuum filtered and washed three times with 10 ml portions of defined salts medium minus glucose at 4 °C. The membranes were dissolved in 10 ml of scintillation fluid (5.5 g of 2.5-diphenyloxazole, 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyle)-benzene per 666 ml of toluene and 334 ml of Triton X-100) and radioactivity was measured in a Model 3320 Packard Tricarb spectrometer.

TABLE II

ATP CONCENTRATIONS IN CELLS GROWING EXPONENTIALLY AT 37 °C WITH AIR AS THE GAS PHASE, AND RELATIONSHIPS AMONG ABSORBANCE, DRY WEIGHT AND COLONY-FORMING UNITS/ml

Details of extraction and assay methods are given in Materials and Methods. Mean ±S.D. are shown for the following number of samples: dry weight, 5; absorbance (glucose) 50; absorbance (succinate) 10; ATP (glucose 121) and ATP (succinate), 34.

	Carbon and energy source	
	Glucose	Succinate
c.f.u. $\times 10^{-8}$ per ml of 1.00 absorbance at 500 nm wavelength	3.07±0.33	7.20±0.71
Dry weight (μg $\times 10^{-7}$ per c.f.u.)	7.65±0.45	–
ATP (molecules $\times 10^{-6}$ per c.f.u.)	1.50±0.16	0.75±0.06
ATP (μmol per g dry weight)	3.26±0.35	3.82±0.31

TABLE III

REDUCTION AND PARTIAL RECOVERY OF ATP CONTENT IN *E. COLI* EXPOSED FOR 2 h TO HYPERBARIC OXYGEN

Cells were exposed in defined salts medium with glucose as the sole carbon source to air (control) or to a mixture of 1 atm of air plus 4 atm of oxygen at 37 °C, then reincubated with air as the gas phase (recovery). ATP was extracted with neutral dimethylsulfoxide at 23 °C for 3 min or with $HClO_4$ (see Table I) and analyzed as described in Materials and Methods. The mean ±S.D. are shown for triplicate determinations of duplicate extractions for the number of experiments shown in parentheses. Dimethylsulfoxide and $HClO_4$ extractions were done on cultures from separate experiments.

Treatment	Time (min)	ATP (molecules $\times 10^{-6}$/cell)	
		Dimethylsulfoxide extraction	$HClO_4$ extraction
Control	–	1.47±0.13 (2)	1.50±0.16 (20)
Hyperbaric O_2	30	0.70±0.11 (2)	0.73±0.07 (4)*
Hyperbaric O_2	45	0.71±0.03 (1)	–
Hyperbaric O_2	60	1.02±0.02 (2)	1.20±0.06 (3)*
Hyperbaric O_2	90	–	1.20±0.07 (1)*
Hyperbaric O_2	120	–	0.21±0.01 (1)*
Recovery after 2 h in O_2	30	–	0.56±0.03 (2)*
Recovery after 2 h in O_2	60	–	1.29±0.10 (2)*
Recovery after 1 h in O_2	5	1.23±0.02 (2)*	1.19±0.04 (2)*
Recovery after 1 h in O_2	30	1.19±0.01 (2)*	1.14±0.04 (2)*
Recovery after 1 h in O_2	60	1.28±0.12 (2)*	0.96±0.03 (2)*
Recovery after 1 h in O_2	75	1.25±0.06 (2)*	–

* Significantly lower than control at $P < 0.05$.

TABLE IV

REDUCTION AND RECOVERY IN ATP CONTENT IN *E. COLI* EXPOSED FOR 30 min TO HYPERBARIC OXYGEN

Experimental details were as described for Table III.

Treatment	Time (min)	ATP (molecules $\times 10^{-6}$/cell)
Control[a]	–	1.50+0.16
Hyberbaric O_2[b]	5	1.68+0.23[c]
	30	0.92±0.09[c]
Recovery[b]	5	0.93±0.07[c]
	15	0.98±0.09[c]
	30	1.41±0.32

[a] The mean ±S.D. for 121 determinations of 20 experiments.

[b] The mean ±S.D. are shown for two experiments with three determinations of each of duplicate extractions for each treatment.

[c] Significantly different from control at $P < 0.05$.

TABLE V

COMPARATIVE EFFECTS OF 15 min OF HYPERBARIC OXYGEN ON THE ATP CONTENT OF *E. COLI* GROWN WITH GLUCOSE OR WITH SUCCINATE AS THE SOLE CARBON AND ENERGY SOURCE

Experimental details were as described for Table III. Mean ±S.D. for two experiments with glucose-grown cells and for three experiments with succinate-grown cells, each with triplicate determinations of duplicate extractions of each treatment.

Treatment	Time (min)	ATP (molecules $\times 10^{-15}$/mg dry cell weight)	
		Glucose-grown	Succinate grown
Control	–	1.93±0.20	2.33±0.13
Hyperbaric O_2	1	2.94±0.18*	2.94±0.15*
Hyperbaric O_2	3	2.64±0.24*	3.21±0.21*
Hyperbaric O_2	5	2.63±0.08*	3.07±0.15*
Hyperbaric O_2	10	1.89±0.14	2.36±0.15
Hyperbaric O_2	15	1.56±0.09*	1.71±0.12*

* Significantly different from control at $P < 0.05$.

Table V also contains comparative ATP values for cells using succinate as the sole carbon and energy source. The results with cells similarly exposed for 30 min to pressure per se, using a mixture of air and nitrogen, are shown in Table VI.

Energy source-deprived cells contained approximately one-third of the ATP/cell present in cells from cultures in exponential growth at 37 °C with glucose as the energy source and air as the gas phase (Fig. 3). ATP was maintained at a relatively constant amount during 30 min of incubation at 37 °C without an energy source, and ATP rapidly accumulated when glucose was added.

TABLE VI

EFFECT OF PRESSURE, PER SE, ON THE ATP CONTENT OF *E. COLI*

Experimental details were as described for Table III, except that the cells were exposed to a mixture of 1 atm of air plus 4 atm of nitrogen. The mean ±S.D. are shown for two experiments, each with three determinations of each of duplicate extractions for each treatment except for the 15- and 23-min treatments which were from one experiment.

Treatment	Time (min)	ATP (molecules $\times 10^{-6}$/cell)
Control	–	1.62±0.08
Hyperbaric O_2	5	1.99±0.20*
Hyperbaric O_2	30	1.89±0.25*
Recovery	5	1.44±0.21*
Recovery	15	1.68±0.21
Recovery	23	1.50±0.11
Recovery	30	1.64±0.30

* Significantly different from control at $P < 0.05$.

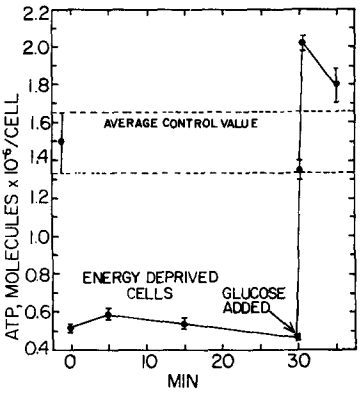

Fig. 3. The effect of energy source deprivation on ATP/cell, and the rate of restoration of ATP following addition of glucose. Cells in exponential growth in defined medium with $2.7 \cdot 10^{-2}$ M glucose as the sole carbon and energy source were washed free of glucose by centrifugation and resuspension three times in the same medium minus glucose. Samples were extracted with $HClO_4$ and analyzed for ATP before and after addition of glucose to a final concentration of $2.7 \cdot 10^{-2}$ M with incubation at 37 °C.

DISCUSSION

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 (Fig. 1), or by incorporation of [^{14}C] glucose into cells (Fig. 2), but did result in an increase in the cellular ATP concentration (Table VI). This increase was greatest after 5 min and was still present after 30 min of exposure. Thus, pressurization per se did not cause a decrease in the ATP content of *E. coli*, but rather a slight increase.

During 2 h of hyperbaric oxygen exposure with ATP determinations at intervals (Table III), the ATP/cell ratio fluctuated; first decreasing, then increasing, and finally decreasing again. These results would occur if the relative inhibition by hyperbaric oxygen of ATP-requiring 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 h (Table III) and was similar to the amount in energy source-deprived cells (Fig. 3). Upon incubation with air as the gas phase, following 1 or 2 h in hyperbaric oxygen (Table III), the ATP/cell increased slowly, compared to the increase observed after adding glucose to energy source-deprived cells (Fig. 3).

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 (Table IV). 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 (Fig. 3) due to a different mechanism (energy source deprivation).

Brief exposure to hyperbaric oxygen (Table V) 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 significantly 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 exposure (Tables IV and V) was also seen during hyperbaric nitrogen exposure with 0.2 atm of oxygen present (Table VI). This initial increase in ATP per cell (Table V) with *E. coli* is in agreement with Chance's findings [12] 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 (Figs. 1 and 2). A significant decrease in cellular ATP did not occur until approx. 15 min (Table V); 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 (Table V). 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 in ATP/cell at 15 min. The overshoots observed upon pressurization (Tables V and VI) and upon addition of glucose to energy source-deprived cells (Fig. 3) are consistent with previously reported conclusions that ATP concentration in *E. coli* is not closely regulated [16].

Sanders et al. [9] 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 [9] which did not occur when the animals received an injection of 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 ATP concentration by succinate [9]. However, we found no indication that succinate protected *E. coli* from the toxic effects of oxygen as measured by the effect on ATP (Table III).

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-h 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.

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REFERENCES

1 Haugaard, N. (1969) Physiol. Rev. 48, 311–373
2 Gottlieb, S. F. (1971) Annu. Rev. Microbiol. 25, 111–152

3 Brown, O. R. (1972) Microbios 5, 7–16
4 Brown, O. R. (1971) J. Bioenerg. 2, 217–220
5 Brunker, R. L. and Brown, O. R. (1971) Microbios 4, 183–203
6 Brown, O. R. and Howitt, H. F. (1969) Microbios 3, 241–246
7 Joanny, P., Corriol, J. and Brue, F. (1970) Science 167, 1508–1510
8 Sanders, A. P., Hall, I. H., Cavanaugh, P. J. and Woodhall, B. (1966) Proc. Soc. Exp. Biol. Med. 121, 32–34.
9 Sanders, A. P., Hall, I. H. and Woodhall, B. (1965) Science 150, 1830–1831
10 Currie, W. D., Pratt, P. C. and Sanders, A. P. (1974) in Oxygen, Fifth International Hyperbaric Congress Proceedings (Trapp, W. G., Barrister, E. W., Davison, A. J. and Trapp, P. A., eds.), Vol. 1, pp. 84–92, Simon Fraser University, Brunaby 2, B.C., Canada
11 Chance, B., Jamieson, D. and Coles, H. (1965) Nature 206, 257–263
12 Chance, B. (1970) Behav. Sci. 15, 1–23
13 Allen, J. E., Goodman, D. B. P., Besarab, A. and Rasmussen, H. (1973) Biochim. Biophys. Acta 320, 708–728
14 Faiman, M. D. and Nolan, R. J. (1974) in Oxygen, Fifth International Hyperbaric Congress Proceedings (Trapp, W. G., Barrister, E. W., Davison, A. J. and Trapp, P. A., eds.), Vol. 1, pp. 156–164, Simon Fraser University, Burnaby 2, B.C., Canada
15 Bagnara, A. S. and Finch, L. R. (1972) Anal. Biochem. 45, 24–34
16 Cole, H. A., Wimpenny, J. W. and Hughes, D. E. (1967) Biochim. Biophys. Acta 143, 445–453