Biochemical Adaptation in Rat Liver in Response to Marginal Oxygen Toxicity

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1. Hepatic glucose 6-phosphate dehydrogenase activity was increased in rats exposed to 5 lb/in² (equivalent to 27000 ft), 100% O₂ when compared with control animals in a 14.7 lb/in² (sea level), air environment. Glyceraldehyde 3-phosphate dehydrogenase, isocitrate dehydrogenase, and succinate dehydrogenase were not affected by the 5 lb/in², 100% O₂ environment. 2. Animals exposed to the hyperoxic environment consumed food, expired CO₂ and gained weight at the same rate as normoxic control animals. Additionally, blood glucose and liver glycogen concentrations were unchanged in the hyperoxic animals. The only readily apparent physiological difference in the hyperoxic animals was a decreased haematocrit. 3. The increase in glucose 6-phosphate dehydrogenase was eliminated by the injection of actinomycin D or cycloheximide. 4. Expiration of ¹⁴CO₂ from [1-¹⁴C]glucose was approximately the same in hyperoxic and normoxic rats. However, ¹⁴CO₂ expiration from [6-¹⁴C]glucose was markedly decreased in the animals exposed to the hyperoxic environment. 5. Calculations of the relative importance of the pentose phosphate pathway versus the tricarboxylic acid cycle plus glycolysis indicated that the livers from animals in the 5 lb/in², 100% O₂ environment metabolized twice as much carbohydrate by way of the pentose phosphate pathway as did those from the sea-level air control animals. 6. In livers of rats exposed to 5 lb/in², 100% O₂ the concentrations of pyruvate, citrate and 2-oxoglutarate were increased, that of isocitrate was slightly elevated, whereas the concentrations of succinate, fumarate and malate were decreased. 7. An inactivation of both tricarboxylic acid cycle lipate-containing dehydrogenases, pyruvate and 2-oxoglutarate, under hyperoxic conditions is proposed. 8. The adaptive significance of the induction of glucose 6-phosphate dehydrogenase and the resultant production of NADPH under hyperoxic conditions is discussed.

The dangers associated with exposure to hyperbaric oxygen and its effects on the central nervous system were described in the nineteenth century by Bert (1847). It has since been found that convulsions occur after a latent period that is inversely proportional to the partial pressure of oxygen (Lambertsen, 1959).

Although the underlying mechanism for oxygen toxicity has not been firmly established, experimental results have led to a number of hypotheses. Lipid peroxidation (Kann, Mengel, Smith & Horton, 1964; Zirkle, Mengel, Horton & Duffy, 1965), oxidation of pyridine nucleotides (Chance, Jamieson & Coles, 1965) and free radical formation (Hauggaard, 1968) have all been suggested. However, an increasing amount of information supports the hypothesis that oxygen toxicity effects a breakdown in oxidative metabolism resulting in a decrease in the amount of energy-rich phosphate

bonds available to nervous tissue (Joanny & Corriol, 1970; Currie, Gelein & Sanders, 1970). A decrease in ATP synthesis in turn leads to the failure of other physiological systems throughout the animal (Brown & Robinson, 1967).

Exposure to hyperbaric oxygen results in the inactivation of several key enzymes of the tricarboxylic acid cycle in rat brain and liver (Thomas, Neptune & Sudduth, 1963; Stadie & Hauggaard, 1945).

Metabolic changes resulting from exposure to oxygen at low pressure are less pronounced, but still detectable. Marked metabolic effects have been reported from this laboratory at values as low as 259 mmHg 100% O₂ (Jordan, Allred, Cahill & Clark, 1966; Bond, Jordan & Allred, 1967). Additional whole-animal investigations in rats at 259 mmHg 100% O₂ have indicated the rate of conversion of [1-¹⁴C]acetate, [6-¹⁴C]glucose and

[2-¹⁴C]pyruvate into ¹⁴CO₂ is significantly decreased (*Status Report*, April, 1967; NASA Grant NsG 300).

In the present paper results are given that show metabolic adaptation in the livers of rats exposed to an environment of 5 lb/in², 100% O₂.

MATERIALS AND METHODS

Reagents. Actinomycin D, cycloheximide, 2-oxoglutarate (disodium salt), isocitrate (trisodium salt), citrate (trisodium salt), pyruvate (monosodium salt), succinate (disodium salt), D-glucose 6-phosphate (disodium salt), D-fructose 1,6-diphosphate (trisodium salt), NAD⁺, NADP⁺ and human serum albumin were obtained from Sigma Chemical Company, St Louis, Mo., U.S.A. Aldolase (10 mg/ml) was purchased from Boehringer Mannheim Corp., San Francisco, Calif., U.S.A. Glucose oxidase (Glucostat) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Dowex 1 (Cl⁻ form) and Dowex 50 (H⁺ form) ion-exchange resins were obtained from California Biochemical Company, Los Angeles, Calif., U.S.A. Boron trifluoride-methanol was purchased from Applied Science Labs, State College, Pa., U.S.A. [1-¹⁴C]Glucose (0.03 mCi/mg) and [6-¹⁴C]glucose (0.27 mCi/mg) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. All other solutions were prepared with analytical-grade reagents obtained from J. T. Baker Company, Phillipsburg, N.J., U.S.A.

Animals. Male albino Holtzman rats, purchased from the Holtzman Company, Madison, Wis., U.S.A., were used in all experiments. Animals were housed in wire cages, and were given water and fed Purina laboratory chow *ad libitum*. Water contained a subclinical dosage of tylosin tartrate (0.5 g/gal) to minimize potential respiratory infections.

At 2 weeks before any experiment, rats were transferred to metabolic chambers at sea-level pressure (14.7 lb/in²) to allow the animals to adapt to living in a closed-chamber environment. During both the acclimatization and experimental phases of the research, food and water were supplied *ad libitum* as described above.

Experimental design. The same basic design was used in all experiments. Between 10 and 12 rats (220–230 g) were placed in a metabolic chamber (at 14.7 lb/in²) 2 weeks before the initiation of experiments. After the 2 week acclimatization period, half of the animals remained in air at 14.7 lb/in² (sea level) and the other half were placed into an identical metabolic chamber in 100% O₂ at 5 lb/in² (27 000 ft). Once the animals were placed in the 100% O₂ atmosphere they remained in it for the duration of the experiment until death. Parallel sea-level air controls were maintained in all experiments.

Chamber system. The metabolic chambers provide a closed ecological system. The system possesses four distinctly different sections. These include (1) the temperature control system to maintain the animals within the chambers at a constant temperature, (2) a constant pressure animal chamber and altitude control system, (3) the negative pressure system which brings the effluent from the animal chamber through the 'scrubber train', which traps CO₂ in 3M-KOH, and removes excess of water with a conc. H₂SO₄ trap, and (4)

the positive-pressure system which reconcentrates the cleaned gases in a pressure-volume reserve tank, feeding them into a gas-mixing tank in which the composition of the gas is monitored and kept constant by a gas-analysis control system. A detailed description of the chamber system has been reported (Kollias & Jordan, 1965; *Status Report*, April, 1967; NASA Grant NsG 300).

Conditions were maintained inside both the experimental and control chambers as follows: (1) the temperature was 24 ± 1°C; (2) gas flow was 1 litre/min per animal; (3) the humidity ranged between 60 and 70%. Total pressure, temperature, water consumption, gas flow rate, oxygen consumption and CO₂ production in both the chambers were continuously monitored and recorded.

Tissue preparation. The rats were killed by decapitation and bled. The experimental animals were killed at the equivalent of 27 000 ft (5 lb/in²), in 100% O₂. Control animals were killed in air at sea level (14.7 lb/in²). The carcasses and blood were immediately passed out of the chamber through an air lock. The livers were excised, blotted, trimmed of excess of fat and weighed. A 2 g piece was cut from the liver for subsequent analysis of soluble-fraction enzymes, and an additional 2 g piece was removed for mitochondrial enzyme analysis. The remainder of the liver was frozen in liquid N₂ and stored at -30°C. The 2 g sample for the soluble-fraction enzyme analysis was homogenized in 20 ml of cold 0.14 M-KCl in a Potter-Elvehjem glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 78 500g for 30 min in a Beckman model L-340 preparative ultracentrifuge.

The 2 g sample for mitochondrial enzymes was homogenized in 10 vol. of cold 0.25 M-sucrose (containing 5 mM-EDTA in a Potter-Elvehjem glass homogenizer with a Teflon pestle. Mitochondrial fractions were obtained by differential centrifugation as described by Schneider & Hogeboom (1950), and were washed once with homogenizing medium and resuspended in 2 ml of the medium.

Blood was analysed for packed erythrocyte volume (haematocrit) and blood glucose concentration. Blood glucose was determined by the glucose oxidase method (Teller, 1956).

Liver glycogen was determined by the anthrone method (Carroll, Longley & Roe, 1956), with glycogen as the standard.

Reaction mixtures. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49), isocitrate dehydrogenase (soluble) (EC 1.1.1.42) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) were assayed by the methods of Freedland (1967). Enzyme activities are expressed as nmol of NAD⁺ or NADP⁺ reduced/min per mg of protein at 25°C.

Succinate dehydrogenase (EC 1.3.99.1) was determined by the dichlorophenol-indophenol method of Green, Mii & Kohout (1955). The assay mixture contained 50 μmol of potassium phosphate buffer, pH 7.4, 1.5 mg of human serum albumin, 6.0 μmol of KCN (freshly neutralized), 0.04 μmol of dichlorophenol-indophenol, 1–2 mg of mitochondrial protein and 50 μmol of succinate. Enzyme activity is expressed as m-units/mg of protein, 1 unit being 1 μmol of dichlorophenol-indophenol reduced/min at 38°C.

Protein determinations. The method of Lowry, Rosebrough, Farr & Randall (1951) was used for the

soluble enzyme preparations. The mitochondrial protein concentration was determined by the biuret method (Gornall, Bardawill & David, 1949).

Collection and determination by scintillation counting of $^{14}\text{CO}_2$. $^{14}\text{CO}_2$ expiration from $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[6\text{-}^{14}\text{C}]\text{glucose}$ was monitored by the following technique. Three animals from the control chamber and three from the experimental chamber were selected at random, weighed and injected intraperitoneally with the appropriate labelled substrate ($150\text{ }\mu\text{Ci/kg}$ body weight). The animals were immediately replaced into their respective chambers for 3 h before being killed. During that time, samples of the $^{14}\text{CO}_2$ expired were obtained by removing portions from the 3M-KOH traps in the 'scrubber train' every 10 min for the first hour, and every 20 min during the second and third hours.

A 0.1 ml portion of the sample was placed into a counting vial and 10 ml of thixotropic gel and 15 ml of dioxan-1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (Bond *et al.* 1967) were added. Each vial was then capped, shaken vigorously with a mechanical stirrer, placed in a liquid-scintillation spectrometer (Packard Tri-Carb) and the radioactivity was counted for three 10 min periods. The radioactivities of duplicate samples were counted for each 60 and 120 min collection periods. The resultant c.p.m. were corrected for efficiency and are reported as d.p.m.

Calculation of pentose phosphate pathway activity. By using the results obtained with $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[6\text{-}^{14}\text{C}]\text{glucose}$, the relative amounts of carbohydrate metabolized through the pentose phosphate pathway compared with the glycolytic pathway (Landau & Katz, 1965) can be calculated. A correction of the $^{14}\text{CO}_2$ results, to account for the lack of complete isomerization by triose phosphate isomerase (EC 5.3.1.1), was made by measuring the incorporation of each substrate into glycerol by a modification of the procedure of Katz, Landau & Bartsch (1966). Since dihydroxyacetone phosphate is a preferential precursor of glycerol, and glyceraldehyde 3-phosphate is preferentially incorporated into fatty acids, the $^{14}\text{CO}_2$ out-put results are normalized by utilizing the following ratio:

$$\delta = \frac{^{14}\text{C in glycerol from } [1\text{-}^{14}\text{C}]\text{glucose}}{^{14}\text{C in glycerol from } [6\text{-}^{14}\text{C}]\text{glucose}}$$

If the assumption is made that $[1\text{-}^{14}\text{C}]\text{glucose}$ can be catabolized to $^{14}\text{CO}_2$ via the pentose phosphate pathway as well as the glycolytic pathway and the tricarboxylic acid cycle, whereas radiolabel from $[6\text{-}^{14}\text{C}]\text{glucose}$ appears in $^{14}\text{CO}_2$ only as the result of metabolism via the glycolytic pathway plus the tricarboxylic acid cycle, then the relative importance of the pentose phosphate pathway can be calculated as:

$$\frac{^{14}\text{CO}_2 (G_1) - 1/\delta \text{ } ^{14}\text{CO}_2 (G_6)}{^{14}\text{CO}_2 (G_1)} \times 100\%$$

where $^{14}\text{CO}_2 (G_1)$ and $^{14}\text{CO}_2 (G_6)$ represent the total amount of $^{14}\text{CO}_2$ expired over a 3 h period from injected $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[6\text{-}^{14}\text{C}]\text{glucose}$, respectively.

Incorporation of ^{14}C into glycerol and total lipid. The incorporation of ^{14}C from $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[6\text{-}^{14}\text{C}]\text{glucose}$ into glycerol was determined after homogenization

of 5 g of tissue and extraction of the lipids with chloroform-methanol-water (Bligh & Dyer, 1959).

Determination of incorporation of ^{14}C into total lipid was made by taking a weighed sample of the lipid, placing it into a counting vial, and dissolving it in 15 ml of toluene-1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene scintillation fluid. The radioactivity in the lipid was corrected for counting efficiency and reported as d.p.m./mg of lipid.

The lipid was saponified for 4 h at 100°C as outlined by Abraham, Matthes & Chaikoff (1960) in the presence of 5 ml of 5M-KOH and 2.5 ml of 95% (v/v) ethanol. After extracting the reaction mixture with *n*-hexane, the saponifiable lipids were obtained by acidifying the remaining water phase to below pH 2 and re-extracting with 3 vol. of *n*-hexane. The extracts were then washed with 0.1M-HCl for the saponifiable fraction.

The aqueous phase, which contained the glycerol, was then evaporated to dryness and extracted with 10 ml of ethanol. A portion of this extract was placed in a counting vial, evaporated to dryness and the radioactivity counted in the presence of 15 ml of dioxane-1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene scintillation fluid. Before the addition of scintillation fluid the amount of glycerol in each vial was determined gravimetrically. The radioactivity in glycerol was corrected for counting efficiency and reported as d.p.m./mg of glycerol. The isolation of the glycerol fraction was necessary to correct the $^{14}\text{CO}_2$ results from $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[6\text{-}^{14}\text{C}]\text{glucose}$ for the calculation of the relative importance of the pentose phosphate pathway compared with glycolysis plus the tricarboxylic acid cycle.

Protein inhibitors. The actinomycin D dosage was $100\text{ }\mu\text{g/kg}$ body wt. (Johnson & Sassoon, 1966) and the cycloheximide dosage was $750\text{ }\mu\text{g/kg}$ body wt. (Bitman, Trezise & Cecil, 1966). Actinomycin D was dissolved in propylene glycol, and cycloheximide in 0.15M-NaCl . Sea-level air controls were sham-injected with 0.35 ml of propylene glycol in the actinomycin D series, and with 0.35 ml of 0.15M-NaCl in the cycloheximide experiment. Antibiotic injections were made at zero time, 1 day and 2 days of exposure to the 51 lb/in^2 , 100% O_2 environment. The animals were killed and the appropriate analyses were performed 180 min after the final (2 day) injection of the appropriate antibiotic.

Gas-liquid chromatography of tricarboxylic acid cycle intermediates. G.l.c. was used to separate and measure the amounts of pyruvate and tricarboxylic acid cycle intermediates in rat liver as described by Kuksis & Prieseschi (1967). After a 48 h exposure to the 51 lb/in^2 , 100% O_2 environment, the rats were decapitated and the entire liver removed and immediately frozen in liquid N_2 . The frozen tissue was then homogenized in HClO_4 , and purified by sequential passage through Dowex 1 (formate form) and Dowex 50 (H^+ form) ion-exchange columns.

The purified liver extracts were then methylated by using 30 ml of 14% (w/v) boron trifluoride-methanol and subsequent boiling for 2 min on a steam bath. The resulting esters were extracted from the water phase with *n*-hexane. The hexane was then removed under N_2 and the esters were diluted to $20\text{ }\mu\text{l}$ with benzene. The esters in benzene were injected into a Varian Aerograph Model 600D hydrogen flame ionization gas chromatograph.

Pyruvate, 2-oxoglutarate, succinate, and fumarate esters were separated by using a 5-ft Carbowax 400M on

Porasil C column. Citrate, isocitrate and malate were separated by using an 8-ft Carbowax 20M 3% (w/w) on Teflon 6 powder (60-80 mesh) column. Both columns used N₂ as the carrier gas with a flow rate of 40 ml/min. The Carbowax 400M column was operated at 135°C for the separation and elution of pyruvate, succinate, and fumarate esters. 2-Oxoglutarate was eluted by raising the column temperature to 160°C. The Carbowax 20M column was operated at 200°C for the elution of citrate, isocitrate, and malate.

RESULTS

Several physiological parameters were measured during the 2 weeks of exposure (Table 1). Neither liver glycogen nor blood glucose concentrations in the experimental animals deviated significantly from sea-level air controls. There is no evidence to indicate a change in glucose utilization or glycogenolysis in the hyperoxic animals. Haematocrits were significantly depressed in the experimental animals after 7 days of exposure to the 5 lb/in², 100% O₂ environment. A decrease in haematocrit without a change in blood volume has been reported during adaptation to 5 lb/in², 100% O₂ (Bartek, Roberts & Ulvedal, 1967). Food consumption was unchanged in the hyperoxic animals, which is consistent with the fact that hyperoxic animals gain weight at approximately the same rate as air control animals, indicating that the experimental animals were maintaining a positive nitrogen balance (Table 1). Of more significance is the fact that CO₂ production from the hyperoxic animals was the same as CO₂ production in air controls (21.62 and 20.98 litres/kg body wt. per day respectively), showing that the hyperoxic animals were also in bioenergetic balance.

Effects of exposure to the 5 lb/in², 100% O₂ environment on enzymes. The specific activities of glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase (soluble), and glyceraldehyde 3-phosphate dehydrogenase were monitored throughout a 2-week exposure to the 5 lb/in², 100% O₂ environment. Additionally, succinate dehydrogenase activity was determined in experiments of shorter duration (2 days). Parallel controls in air at sea level (14.7 lb/in²) were also monitored throughout the experiments. Only glucose 6-phosphate dehydrogenase showed a significant difference from control animals (Table 2). A significant aspect of the glucose 6-phosphate dehydrogenase results is that the apparent maximal increase in activity occurs during the first 24 h of exposure, and declines over the next 14 days to control values. This indicates the stress responsible for the elevation in glucose 6-phosphate dehydrogenase activity is compensated for by adaptive processes. NADP⁺-dependent isocitrate dehydrogenase is apparently not involved in the adaptation to the stress, its specific activity remaining

Table 1. *Physiological parameters measured during exposure to a 5 lb/in², 100% oxygen environment for 2 weeks*

	Exposure time (days)	Liver glycogen (mg/g of liver)	Blood glucose (mg/100 ml)	Packed erythrocyte volume (haematocrit)	Food consumption (g/day per kg body wt.)	Body weight gain (g/day per kg body wt.)
Experimental	1	33.38 ± 5.62	113.15 ± 8.3	45.03 ± 0.5	76.29	26.68
Control	1	32.36 ± 5.91	119.39 ± 7.8	46.35 ± 0.4	78.31	24.29
Experimental	3	29.31 ± 2.42	120.78 ± 3.5	44.07 ± 0.5	79.29	26.28
Control	3	32.40 ± 3.51	118.64 ± 7.3	45.23 ± 0.3	84.22	28.84
Experimental	7	36.16 ± 4.27	117.66 ± 8.7	42.62 ± 0.6*	85.14	25.89
Control	7	26.36 ± 5.75	122.82 ± 8.3	44.86 ± 0.4	85.99	25.30
Experimental	14	35.08 ± 4.10	125.96 ± 5.8	42.33 ± 0.4*	88.92	22.65
Control	14	31.56 ± 5.35	111.80 ± 3.8	45.27 ± 0.4	89.04	24.09

* $P < 0.01$.

Liver glycogen was determined by the anthrone method (Carroll *et al.* 1955). Blood glucose was determined by the glucose oxidase method (Teller, 1956). Values are given from two identical experiments as means ± s.e.m. for six animals, except food consumption and body weight gain which are means for the two experiments. Experimental refers to animals exposed to a 5 lb/in² (27 000 ft), 100% oxygen environment. Control values are for animals exposed to a 14.7 lb/in² (sea level), air environment.

Table 2. *Enzymic activity during exposure to a 5 lb/in², 100% oxygen environment*

Results are given as nmol of NAD⁺ or NADP⁺ reduced/min per mg of protein for the pyridine nucleotide-linked dehydrogenases. Succinic dehydrogenase activity is given as nmol of dichlorophenol-indophenol reduced/min per mg of protein. Values are means $\pm$ S.E.M. of six observations. For details of assays see the Materials and Methods section.

Enzyme assayed	Exposure time (days)	Experimental (5 lb/in ² , 100% O ₂)	Control (14.7 lb/in ² , air)	P
Glucose 6-phosphate dehydrogenase	1	30.24 $\pm$ 3.69	20.03 $\pm$ 1.76	<0.01
	3	26.57 $\pm$ 3.26	17.30 $\pm$ 0.43	<0.01
	7	21.69 $\pm$ 2.22	17.97 $\pm$ 1.01	N.S.
	14	18.95 $\pm$ 0.29	16.49 $\pm$ 0.93	N.S.
Isocitrate dehydrogenase	1	121.97 $\pm$ 4.64	130.50 $\pm$ 9.92	N.S.
	3	138.76 $\pm$ 4.09	129.32 $\pm$ 5.33	N.S.
	7	131.97 $\pm$ 5.22	136.08 $\pm$ 8.66	N.S.
	14	112.93 $\pm$ 5.52	111.01 $\pm$ 7.54	N.S.
Glyceraldehyde 3-phosphate dehydrogenase	1	111.21 $\pm$ 18.35	96.28 $\pm$ 11.87	N.S.
	3	105.15 $\pm$ 14.17	89.42 $\pm$ 12.71	N.S.
	7	85.34 $\pm$ 10.81	89.63 $\pm$ 11.25	N.S.
	14	88.76 $\pm$ 5.39	79.05 $\pm$ 3.47	N.S.
Succinate dehydrogenase	2	9.89 $\pm$ 1.61	9.84 $\pm$ 0.79	N.S.

unchanged throughout the 2-week exposure. Glyceraldehyde 3-phosphate dehydrogenase was also not affected in this system by the 100% O₂ environment; this was surprising, since it has been shown that this thiol-containing enzyme is probably inactivated by high oxygen 'tensions' (Mengel, Kann, Lewis & Horton, 1964), and may thus be responsible for decreased glycolytic activity. Succinate dehydrogenase activity was monitored because this enzyme has been reported to be sensitive to changes in oxygen 'tensions' (Stadie & Haugaard, 1945; Aithal & Ramasarma, 1969). Succinate dehydrogenase activity did not change in the 5 lb/in², 100% O₂ environment (Table 2).

Protein synthesis inhibitors. The nature of the increase in glucose 6-phosphate dehydrogenase activity in animals exposed to a 5 lb/in², 100% O₂ environment was studied. Since the maximum elevation in activity occurred after 24–48 h in the hyperoxic environment, in experiments with actinomycin D or cycloheximide, these agents were administered during a 2-day exposure. Both actinomycin D and cycloheximide were effective in decreasing the induction of glucose 6-phosphate dehydrogenase (Table 3). These results indicate that synthesis *de novo* of protein is necessary for increased glucose 6-phosphate dehydrogenase activity in hyperoxic conditions.

Because of the many side effects associated with the use of these drugs, and the fact that food intake and hexose monophosphate-shunt activity are so closely connected (Tepperman & Tepperman, 1963), food consumption was carefully monitored. Neither antibiotic appreciably altered food consumption (Table 3).

Injection of [1-¹⁴C]glucose and [6-¹⁴C]glucose. The

importance of the pentose phosphate pathway to carbohydrate metabolism relative to that of glycolysis and the tricarboxylic acid cycle together was investigated by using [1-¹⁴C]glucose and [6-¹⁴C]glucose. The animals exposed to 100% O₂ metabolized [1-¹⁴C]glucose at approximately the same rate as the air control animals (Fig. 1). However, [6-¹⁴C]glucose was metabolized at a significantly slower rate in the animals exposed to 100% oxygen than in the parallel air controls (Fig. 1). When the ¹⁴CO₂ results were corrected as described above, calculations of the relative importance of the pentose phosphate pathway indicate that twice as much carbohydrate was metabolized by the hexose monophosphate shunt in the animals exposed to oxygen as in the control animals (Table 4). The incorporation of [1-¹⁴C]glucose and [6-¹⁴C]glucose into glycerol, on which the calculations of the relative importance of the pentose phosphate pathway are based, are reported in Table 4.

Additionally, the incorporation of [1-¹⁴C]glucose and [6-¹⁴C]glucose into total lipid was not significantly different in experimental animals when compared with sea-level controls (Table 4).

Quantitative analysis of tricarboxylic acid-cycle intermediates. Increased glucose 6-phosphate dehydrogenase activity, and a doubling in the relative importance of the pentose phosphate pathway *in vivo* are conclusive evidence that the hexose monophosphate shunt was activated under hyperoxic conditions. The mechanism responsible for the increase in pentose phosphate pathway activity was originally postulated to be an inactivation of glyceraldehyde 3-phosphate dehydrogenase. However, our results do not support this theory (Table 1). An inactivation of one or more of the enzymes

Table 3. *Glucose 6-phosphate dehydrogenase activity and food consumption after injection of actinomycin D or cycloheximide*

		Actinomycin D		Cycloheximide	
		Experimental (5lb/in ² , 100% O ₂)	Control (14.7lb/in ² , air)	Experimental (5lb/in ² , 100% O ₂)	Control (14.7lb/in ² , air)
		% of control values		% of control values	
		110		106	
		94		88	
		17.50±0.80		18.29±0.79	
		69.67		63.97	
		19.16±0.56		19.29±1.13	
		65.60		55.98	
		Glucose 6-phosphate dehydrogenase activity			
		Food consumption (g/day per kg body wt.)			

Actinomycin D was injected at the rate of 100 µg/kg body wt., and cycloheximide at the rate of 750 µg/kg body wt. Glucose 6-phosphate dehydrogenase activity is expressed as nmol of NADP⁺ reduced/min per mg of protein. Values are the means±s.e.m. of five observations. Values for food consumption are absolute values. Experimental values represent rats exposed to a 5lb/in² (27 000 ft), 100% oxygen environment and injected with the appropriate antibiotic. Control values are from animals exposed to air at 14.7lb/in² (sea level) and sham injected. Injections were made at zero time, 1 day and 2 days exposure, and the animals were killed 180 min after the final injection of antibiotic.

Fig. 1. Accumulative ¹⁴CO₂ expiration from [1-¹⁴C]glucose (0.03 mCi/mg) and [6-¹⁴C]glucose (0.027 mCi/mg). The injected dose of radioactive glucose was 150 µCi/kg body wt. (a) ¹⁴CO₂ from [1-¹⁴C]glucose. (b) ¹⁴CO₂ from [6-¹⁴C]glucose. Points are the means of three observations. The technique of sample collection and liquid-scintillation counting are described in detail in the Materials and Methods section. (○) 5lb/in², 100% O₂, (●) 14.7lb/in², air.

of the tricarboxylic acid cycle would also divert substrates toward the pentose phosphate pathway; therefore, concentrations of tricarboxylic acid-cycle intermediates were determined. The experimental animals had a fourfold increase in pyruvate, twice as much citrate, and slightly increased isocitrate concentrations. Additionally, the hyperoxic animals had a twofold increase in 2-oxoglutarate concentration, and decreased concentrations of succinate, fumarate, and malate when compared with control animals (Table 5). Obviously, exposure to the 5lb/in², 100% O₂ environment results in altered pyruvate and tricarboxylic acid cycle intermediate metabolism. These differences in the concentration of pyruvate and tricarboxylic acid-cycle intermediates in the oxygen-exposed animals are uniformly statistically significant, and indicate strongly that there are probably two blocks in oxidative glucose metabolism in these animals. Since pyruvate and 2-oxoglutarate concentrations are very high, it appears that 2-oxoglutarate de-

Table 4. *Relative importance of the pentose phosphate pathway and [1-¹⁴C]- and [6-¹⁴C]-glucose incorporation into glycerol and total lipids*

Calculations of relative importance of the pentose phosphate pathway against tricarboxylic acid cycle + glycolysis are explained in the Materials and Methods section. Incorporation of [1-¹⁴C]glucose (0.03 mCi/mg) and [6-¹⁴C]glucose (0.027 mCi/mg) into glycerol are expressed as d.p.m./mg of glycerol and values are means $\pm$ S.E.M. of four observations.

	% Glycolysis and tricarboxylic acid cycle	% Pentose phosphate pathway	Incorporation into glycerol		Incorporation into total lipids	
			[1- ¹⁴ C]Glucose	[6- ¹⁴ C]Glucose	[1- ¹⁴ C]Glucose	[6- ¹⁴ C]Glucose
Experimental (5lb/in ² , 100% O ₂)	59.44	40.56	373.64 $\pm$ 37.67	356.85 $\pm$ 6.02	403.39 $\pm$ 111.89	536.38 $\pm$ 53.54
Control (14.7lb/in ² , air)	80.43	19.57	391.78 $\pm$ 30.47	330.70 $\pm$ 36.74	433.25 $\pm$ 50.03	646.07 $\pm$ 31.76

Table 5. *Concentrations of pyruvate and tricarboxylic acid-cycle intermediates after hyperoxic exposure*

Values are presented as μ mol of acid/100 g of liver. All results are means $\pm$ S.E.M. of four observations. Actual measurement of tricarboxylic acid cycle intermediates was accomplished by g.l.c. (see the Materials and Methods section for details).

Compound	Experimental (5lb/in ² , 100% O ₂)	Control (14.7lb/in ² air)	% of control	P
Pyruvate	131.13 $\pm$ 21.36	33.98 $\pm$ 3.18	386	<0.005
Citrate	32.63 $\pm$ 3.84	13.95 $\pm$ 0.84	235	<0.005
Isocitrate	4.11 $\pm$ 0.53	3.12 $\pm$ 0.16	132	<0.025
2-Oxoglutarate	28.87 $\pm$ 2.60	18.13 $\pm$ 1.80	159	<0.010
Succinate	14.83 $\pm$ 0.83	25.66 $\pm$ 1.75	58	<0.005
Fumarate	13.08 $\pm$ 1.33	22.58 $\pm$ 1.33	58	<0.005
Malate	8.39 $\pm$ 1.15	16.85 $\pm$ 1.69	49	<0.005

hydrogenase and pyruvate dehydrogenase may both be inactivated by the 5lb/in², 100% O₂ environment.

DISCUSSION

Although animals exposed to the 5lb/in², 100% O₂ environment were in bioenergetic balance, the increased glucose 6-phosphate dehydrogenase activity, the doubling in the relative importance of the pentose phosphate pathway, and the decreased expiration of [6-¹⁴C]glucose by the hyperoxic animals are substantial evidence for altered oxidative glucose metabolism. Although a prime suspect, glyceraldehyde 3-phosphate dehydrogenase activity was not altered. The increased concentrations of pyruvate, citrate and 2-oxoglutarate and the low concentrations of succinate, fumarate and malate in the liver of the hyperoxic animals suggest that both lipoyl-containing dehydrogenases, 2-oxoglutarate and pyruvate, may be inactivated by the marginally hyperoxic environment. This hypothesis is further supported by results from our laboratory (R. R. Gorman, J. P. Jordan, J. B. Simmons & D. P. Clarkson,

unpublished work) showing that the injection of tricarboxylic acid-cycle intermediates such as succinate will decrease the induction of glucose 6-phosphate dehydrogenase whereas injection of 2-oxoglutarate actually potentiates the increase in glucose 6-phosphate dehydrogenase activity.

A decrease in tricarboxylic acid-cycle activity would substantially decrease the adenylate energy charge of the cell (Atkinson, 1968). An apparent decrease in oxidative phosphorylation has been reported in rats exposed to pure oxygen at 380 mm Hg (Brown & Robinson, 1967). However, the decrease was measured *in vitro*, and adaptation under these conditions was impossible. A decrease in available ATP would undoubtedly result in loss of weight and bioenergetic imbalance in the absence of compensation. The animals in this study maintained bioenergetic balance, indicating that adaptation did occur, and the adenylate energy charge of the cell was maintained. We attribute increased glucose 6-phosphate dehydrogenase activity to a need for an extramitochondrial supply of reducing power for ATP production. The hexose monophosphate shunt provides such a source

of reducing equivalents. The transfer of these reducing equivalents from the cytosol to the mitochondrial electron transport system and at the same time circumventing blocks in the tricarboxylic acid cycle might be accomplished through the use of the 'malate shuttle' (Simpson & Estabrook, 1969). 'Malic' enzyme requires NADPH, CO_2 and pyruvate, all which are in plentiful supply in this system. Although glucose 6-phosphate dehydrogenase activity was measured in the liver only, the maintenance of bioenergetic balance by rats in 5lb/in^2 , 100% O_2 indicates that other tissues of the body also adapt to the hyperoxic environment.

Through the use of the 'malate shuttle' the animal might be able to supply reducing equivalents to the electron transport system within the mitochondrion, thereby maintaining the adenylate energy charge of the cell. As other physiological adaptations occur that lower the $p\text{O}_2$ at the mitochondrion (e.g., decreased haematocrit), this shuttle would decrease in importance until normal metabolism is restored. In very high pressures of pure oxygen, the malate shuttle probably is not sufficient to maintain the concentration of ATP; the animal is unable to adapt and dies.

Important to the hypothesis that the increased glucose 6-phosphate dehydrogenase activity is a source of reducing equivalents for ATP production and not the result of increased biosynthetic activity is the fact that fatty acid synthesis in these animals exposed to oxygen is actually significantly depressed (Jordan *et al.* 1966; Bond *et al.* 1967). Obviously, increased fatty acid synthesis does not accompany the increased pentose phosphate pathway activity.

Although glucose 6-phosphate dehydrogenase activity is increased under hyperoxic conditions, the findings are against a direct effect of oxygen on the enzyme itself. Our evidence indicates that the underlying mechanism for oxygen toxicity in the 5lb/in^2 , 100% O_2 environment may be the same as that reported by Thomas *et al.* (1963) for brain tissue in high pressure O_2 , namely inhibition of pyruvate and 2-oxoglutarate dehydrogenases.

CoA concentrations are not appreciably decreased in rats exposed to 5lb/in^2 , 100% O_2 until after 14 days of exposure (D. V. Lassiter, unpublished work), so that CoA is probably not involved in the inactivation of the dehydrogenases. The inhibition could be mediated through the direct oxidation of thiol groups associated with the enzyme complexes, or through the formation of glutathione-protein disulphide bridges as postulated by Jocalyn (1960).

The only apparent difference between high pressure and low pressure oxygen toxicity is that the effect is not so severe in the low pressure O_2 environment, and the animal is able to adapt, where-

as oxygen at partial pressures above 500 mm Hg rapidly leads to death in rats (Dickerson, 1964).

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REFERENCES

- Abraham, S., Matthes, K. J. & Chaikoff, I. L. (1960). *J. biol. Chem.* **235**, 2551.
- Aithal, H. N. & Ramasarma, T. (1969). *Biochem. J.* **115**, 77.
- Atkinson, D. E. (1968). *Biochemistry*, Easton, **7**, 4030.
- Bartek, M. J., Roberts, A. J. & Ulvedal, F. (1967). *Aerospace Med.* **38**, 1037.
- Bert, P. (1847). *La Pression Barometrique*. Paris: Masson. Translated by Hitchcock, F. (1943). *Barometric Pressure*. Columbus, Ohio: London College Book Co.
- Bitman, J., Trezise, L. A. & Cecil, H. C. (1966). *Archs Biochem. Biophys.* **114**, 414.
- Bligh, E. G. & Dyer, W. J. (1959). *Can. J. Biochem. Physiol.* **37**, 911.
- Bond, A. D., Jordan, J. P. & Allred, J. B. (1967). *Am. J. Physiol.* **212**, 252.
- Brown, H. E. & Robinson, C. E. (1967). AF Contract 4(609)-3103, Project-Task no. 793002.
- Carroll, N. V., Longley, R. W. & Roe, J. H. (1956). *J. biol. Chem.* **220**, 583.
- Chance, B., Jamieson, D. & Coles, H. (1965). *Nature, Lond.*, **206**, 257.
- Currie, W. D., Gelein, R. M., jun. & Sanders, A. P. (1970). *Proc. Soc. exp. Biol. Med.* **133**, 103.
- Dickerson, K. H. (1964). NADC-ML-6403, U.S. Naval Air Development Center.
- Freedland, R. A. (1967). *J. Nutr.* **9**, 489.
- Gornall, A. G., Bardawill, G. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 75.
- Green, D. E., Mii, S. & Kohout, D. M. (1955). *J. biol. Chem.* **217**, 651.
- Haugaard, N. (1968). *Physiol. Rev.* **48**, 311.
- Joanny, P. & Corriol, J. (1970). *Science, N.Y.*, **167**, 1508.
- Jocalyn, P. C. (1960). *Biochem. J.* **77**, 368.
- Johnson, B. C. & Sassoon, H. F. (1966). In *Advances in Enzyme Regulation*, vol. 5, p. 93. Ed. by Weber, G. London: Pergamon Press Ltd.
- Jordan, J. P., Allred, J. B., Cahill, C. L. & Clark, R. T. (1966). *Aerospace Med.* **37**, 368.
- Kann, H. E., jun., Mengel, C. E., Smith, W. & Horton, B. (1964). *Aerospace Med.* **35**, 840.
- Katz, J., Landau, B. R. & Bartsch, G. E. (1966). *J. biol. Chem.* **241**, 727.
- Kollias, J. & Jordan, J. P. (1965). *Am. J. appl. Physiol.* **20**, 742.
- Kuksis, A. & Prioreshi, P. (1967). *Analyt. Biochem.* **19**, 468.
- Lambertsen, C. J. (1959). *Publs natn. Acad. Sci.-natn. Res. Council*, no. 377: *Proc. Symp. Underwater Physiology* p. 72.
- Landau, B. R. & Katz, J. (1965). *Handbook of Physiology*. Section 5, p. 253. Washington, D.C.: American Physiological Society.

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mengel, C. E., Kann, H. E., jun., Lewis, A. M. & Horton, B. (1964). *Aerospace Med.* **35**, 857.
- Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
- Simpson, E. & Estabrook, R.W. (1969). In *Advances in Enzyme Regulation*, vol. 7, p. 259. Ed. by Weber, G. London: Pergamon Press Ltd.
- Stadie, W. & Haugaard, N. (1945). *J. biol. Chem.* **161**, 181.
- Teller, J. D. (1956). *Abstr. 130th Meet. Am. chem. Soc.* p. 69 c.
- Tepperman, H. M. & Tepperman, J. (1963). In *Advances in Enzyme Regulation*, vol. 1, p. 121. Ed. by Weber, G. London: Pergamon Press Ltd.
- Thomas, J. J., jun., Neptune, E. M., jun. & Sudduth, H. C. (1963). *Biochem. J.* **88**, 31.
- Zirkle, L. G., jun., Mengel, C. E., Horton, B. D. & Duffy, E. J. (1965). *Aerospace Med.* **36**, 1027.