Effects of Hyperbaric Oxygenation on Metabolism. IV. Time Sequence of Biochemical Changes at 5 Atmospheres 100% Oxygen.* (32188)

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Previous reports have shown that brain, liver and kidney from fasted male Sprague-Dawley rats (150-225 g), which had survived exposures to 5 atmospheres (ATA) of 100% oxygen for 90 minutes, exhibited changes in ATP concentration(1), respiration and oxidative phosphorylation(2), succinic dehydrogenase activity, free acid phosphatase activity, free cathepsin activity, and soluble nitrogen(3,4). Rats subjected to such exposures die during the exposure period, or within 20 minutes after removal from the chamber. These alterations in the tissues indicated the need for establishing the sequence of tissue changes which occur preceding and during acute hyperoxia toxicity.

This paper reports changes in ATP concentration, succinic dehydrogenase activity, % free cathepsin activity, % free ribonuclease activity, total nitrogen, acid soluble nitrogen, and tissue buffer capacity in brain, liver and kidney from Sprague-Dawley rats subjected to 5 ATA of 100% oxygen for 30, 60, and 90 minutes.

Methods. Male Sprague-Dawley rats (150-200 g) were fasted 18 to 24 hours preceding any experiment with water *ad libitum*. Control studies were obtained from animals exposed to air at 1 atmosphere. Other groups of animals were exposed to 100% oxygen at 5 atmospheres pressure for 30, 60, and 90 minutes. An oxygen flow rate (measured at 1 ATA) of greater than 0.5 liters per minute per animal in the HPO chamber was maintained throughout the HPO exposure period. On decompression the tissues were rapidly removed and processed according to the analyses to be performed.

ATP concentration was determined by the firefly luminescence method(1,5-8). The method of Cooperstein, Lazarow, and Kurfess (9) was used to determine succinic dehydrogenase activity. Free and total cathepsin activity was determined by the method of Anson(10) as modified by Press, Porter, and Cebra(11). Free and total ribonuclease activity was determined by the method of Slater(12). Total cathepsin and ribonuclease enzyme activity was released in the homogenate by Triton-X-100 (0.1%)(13). The method of deHaan and Field(14) was used to determine tissue buffer capacity. Acid soluble and total nitrogen was determined by micro-Kjeldahl digestion coupled with Nessler reagent.

Results. The variation in tissue ATP concentration with time of high pressure oxygen (HPO) exposure is shown in Table I. All 3 tissues showed a loss at 30 minutes (29 to 39%), a return to normal in liver and kidney at 60 minutes, and a significant decrease in the concentration of all 3 tissues at 90 minutes (45 to 63%).

Table II shows the variation in tissue buffer capacity with time of HPO exposure. By 30 minutes the 3 tissues showed an alkaline shift that is maintained throughout the 90 minute exposure period.

Fig. 1 shows the variations in succinic dehydrogenase and % free ribonuclease activities as a function of HPO exposure time. There was an inverse relationship between succinic dehydrogenase activity and time of HPO exposure for all 3 tissues. The free ribonuclease activity in brain remained unchanged until the 90 minute exposure period, when the activity was 243% of control values. Kidney showed a significant increase

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TABLE I. ATP Concentration as % of Control.

Tissue	Control	N	100% O_2, 5 ATA exposure 30 min	N	60 min	N	90 min	N
Brain	100 ± 6.0	8	61 ± 10 P <.01	6			47 ± 11 P <.01	8
Liver	100 ± 21	8	71 ± 19.5 P <.01	6	95 ± 25 N.S.	10	55 ± 14 P <.01	5
Kidney	100 ± 14	12	71 ± 18 P <.01	6	126 ± 15 P <.01	8	37 ± 8 P <.01	5

Mean ± standard deviation. N.S. = Not significant.

TABLE II. Tissue Buffer Capacity (ml of 0.1 N HCl Required to Reduce the pH of 2.0 ml of a 10% Homogenate from pH 11.0 to 2.0).

Tissue	Control	N	100% O_2, 5 ATA exposure 30 min	N	60 min	N	90 min	N
Brain	2.04 ± .11	10	2.69 ± .23 P <.01	6	2.65 ± .43 P <.01	6	2.67 ± .14 P <.01	6
Liver	2.28 ± .26	11	2.74 ± .21 P <.01	6	2.69 ± .36 P = .02	6	2.66 ± .11 P <.01	6
Kidney	1.89 ± .09	12	2.58 ± .18 P <.01	6	2.53 ± .15 P <.01	6	2.63 ± .08 P <.01	6

Mean ± standard deviation.

in free ribonuclease activity at 30, 60, and 90 minutes of HPO exposure—reaching a maximum of 136% of control levels at the 90 minute period. Liver showed significant increases in free ribonuclease activity at 30 and 90 minutes with a maximum of 200% of the control level at the 90 minute point.

The variation in % free cathepsin activity, acid soluble and total nitrogen with time of HPO exposure are shown in Fig. 2. There was a marked increase in the free cathepsin activity with increasing time of exposure in all 3 tissues with the exception of the 90 minute time in kidney. The apparent decrease in kidney at 90 minutes is considered to be due to an inactivation of the enzyme

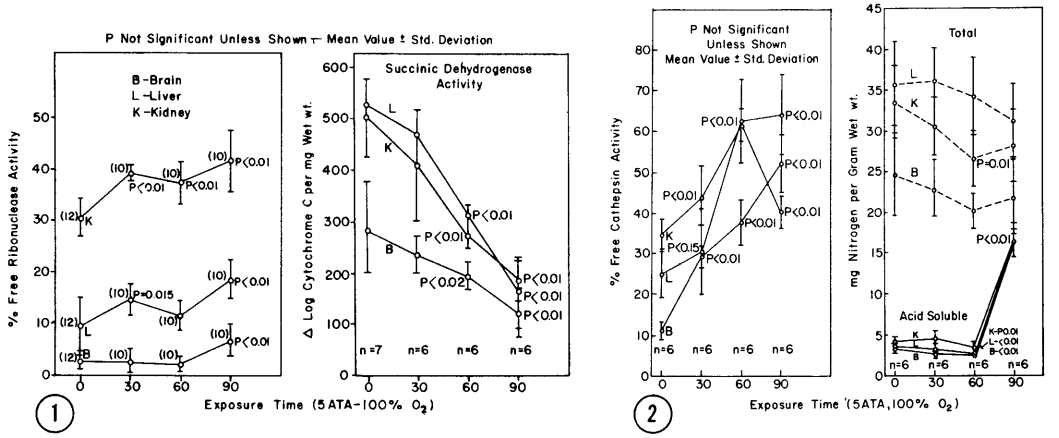

FIG. 1. Variation in % free ribonuclease and succinic dehydrogenase activity with time of HPO exposure.

FIG. 2. Variation in % free cathepsin activity, total and acid soluble nitrogen with time of HPO exposure.

by HPO, with a resultant decrease both in total and free cathepsin activity. The total nitrogen in brain and liver was not significantly different from control values at 30, 60, and 90 minutes HPO exposure. Kidney showed a statistically significant decrease in total nitrogen of 21% at 60 minutes of HPO exposure. The dry weight : wet weight ratio deviated only 12% from the kidney of controls at 60 minutes. We are unable to explain the apparent loss of total nitrogen above that accounted for by the decrease in the drv weight : wet weight ratio. The 30 and 90 minute exposure caused no statistically significant decrease in total nitrogen in kidney. The acid soluble nitrogen was not significantly different from control levels after 30 minutes exposure, decreased after 60 minutes HPO exposure, and markedly increased to 490%, 452%, and 383% of control levels for brain, liver and kidney, respectively, after 90 minutes HPO exposure.

Discussion. The return of ATP concentration toward normal levels at 60 minutes is considered to be a compensatory action due to vascular changes in the tissues, or mitochondrial changes resulting in increased permeability and/or an increased number of functional electron transport chain units. Under conditions of anoxia which result in decreased ATP levels(15,16), mitochondrial swelling(17) and permeability changes(18) have been reported. Such tissues when returned to normal oxygen tensions show increased respiration capacity (relative to normal) and ATP production capacity(16). We have observed that tissues subjected to HPO with demonstrated ATP concentration loss have an increased respiration and ATP production capacity(2). Thus one explanation of the return of ATP concentration toward normal at 60 minutes may be that mitochondrial changes result in increased respiration capacity in these tissues. Between 60 and 90 minutes there is a loss in ATP concentration in brain, liver and kidney (53%, 45%, and 63%, respectively). We have previously shown that at the 90 minute exposure period there is an increased respiration and ATP production capacity in brain and liver, when returned to normal oxygen tensions, and only a 20% reduction in respiration and ATP production capacity in kidney(2). Thus the reduced ATP concentration cannot be due to the destructive action of the increased free cathepsin on mitochondria, rendering the mitochondria non-functional. The decreased ATP concentrations must be a result of HPO rendering the mitochondrial respiratory chain units (dehydrogenases, flavoproteins, electron transport chain and cofactors) non-functional, or activating ATPase. Chance *et al*(19) have shown an inhibition of NAD reduction by HPO. Thomas *et al*(20) have demonstrated that HPO causes a rapid inhibition of alpha-ketoglutarate dehydrogenase activity. Either of these two inhibiting actions of HPO could render the mitochondrial respiratory chain units non-functional and result in a loss of ATP production. It is conceivable that it takes between 60 and 90 minutes of exposure to 100% oxygen at 5 ATA before HPO affects a sufficient number of respiratory chain units to result in a continuous decrease in ATP concentration.

ATPase activity was determined in brain, liver and kidney from rats exposed to 5 ATA, 100% oxygen for 90 minutes to see if increased ATPase activity was a contributing factor in the decreased ATP concentration found in the 3 tissues after such exposures. Table III shows the results.

All 3 tissues had reduced ATPase activity for this exposure period. Thus the decrease in ATP concentration in the 3 tissues after such HPO exposure cannot be due to increased ATPase activity.

Coinciding with the decrease in ATP concentration between the 60 and 90 minute exposure, was a marked increase in acid soluble nitrogen. It was during this period that rats exhibited symptoms of acute oxygen toxicity. Increases in free cathepsin activity were found in all 3 tissues at 30, 60, and 90 minute HPO exposures. The fact that the acid soluble nitrogen did not significantly increase until the 60 to 90 minute time interval (as evidenced by the 90 minute soluble nitrogen content) suggests that during the 30 and 60 minute exposures the free proteolytic lysosomal enzymes have remained at-

TABLE III. Magnesium Activated ATPase Activity at 37°C (Micromoles of ATP Hydrolyzed per Gram [Wet Weight] per Minute).

	Brain	Liver	Kidney	N
Control	7.25 ± .49	3.84 ± .45	10.2 ± 1.09	10
90 min, 5 ATA, 100% O_2	6.68 ± .72	2.54 ± .57	9.50 ± 1.05	10
	P = .05	P < .01	P = .165	

Mean ± standard deviation. P = Probability being same as the controls.

tached to or in the vicinity of the lysosome. Acid soluble nitrogen increased markedly in the last 30 minutes of the exposure period when there was a concomitant decrease in ATP concentration. This raises the question of a possible inverse relationship between these two entities. Wilhelmi *et al*(21) observed in liver of rats subjected to prolonged periods of anoxia [which have been shown to significantly decrease ATP concentration in tissues(16)], a marked increase in free nitrogen. They hypothesized that when the energy stores of tissue are reduced, energy utilization shifts from anabolism to catabolism in an effort to provide substrate sources for energy production and the maintenance of energy stores. Rat liver subjected to the same periods of anoxia (1 hour) *in vitro* had free cathepsin activity of 487% of controls with no significant increase in acid soluble nitrogen(22). Rat liver subjected to 12 hours autolysis at 37°C had free cathepsin activity 337% of normal and acid soluble nitrogen of only 210% of normal(22). These experiments with HPO exposures of 60 minutes of 100% oxygen, 5 ATA, had liver free cathepsin activity of 250% of normal and acid soluble nitrogen only 75% of normal, and free cathepsin activity of 256% of normal with acid soluble nitrogen being 452% of normal after 90 minutes exposure. Thus it is not possible to conclude that the marked increase in acid soluble nitrogen between the 60 and 90 minute exposure period was only a function of increased free lysosomal proteolytic enzyme activity. In anoxic tissues ATP falls rapidly (within 1 minute) to the stable ATP levels (in liver, brain, and kidney of the rat)(16), thus losing essentially all sources of energy for further cell functions. Under the HPO conditions this very rapid loss of ATP does not occur. ATP was available (47%, 55%, and 37% of normal in brain, liver and kidney, respectively), at the 90 minute exposure time. Thus the more severe increase in acid soluble nitrogen found in these HPO experiments, when compared with the autolysis experiments(22), may be a function of ATP energy being available to facilitate catabolism and/or that HPO may activate enzymes, and/or cofactors in the proteolytic process.

Increases in free lysosomal enzyme activity with HPO exposure has been attributed to HPO acting directly on the lysosome to release the enzymes(23), HPO causing lipid peroxidation with resultant increased permeability of the lysosome membranes(24,25), and intracellular acidity(3). The measurement of tissue buffer capacity of the 3 tissues at 30, 60, and 90 minutes, 5 ATA, 100% oxygen exposure showed a decrease in tissue acidity in the 3 tissues (Table II). If the intracellular changes observed under these conditions are to be attributed to changes in hydrogen ion concentration, then it must be due to a decrease, rather than an increase, in tissue acidity.

The method used for measuring tissue buffer capacity could be questioned in view of the discrepancy between the observed decrease in acidity with HPO exposure and the generally advanced hypothesis that HPO exposure results in increased tissue acidity (3,26). The same method was employed to measure tissue buffer capacity under anoxic conditions, and the results indicated increased acidity in anoxic tissues(14,22). The increased acidity in anoxic tissues had previously been demonstrated with the use of tissue pH electrodes(27). Thus the method for measuring tissue buffer capacity is capable of measuring variation in tissue acidity, and the decreased tissue acidity measured in the

3 tissues from rats exposed to 100% oxygen at 5 ATA for 30, 60, and 90 minutes is considered valid.

The decrease in hydrogen ion concentration in the 3 tissues with HPO exposure is considered to be due to hyperventilation, and subsequent CO_2 elimination, which was observed in these animals. This would imply that a shift in the acidity of extracellular fluid results in a corresponding directional shift in intracellular acidity. These data support this concept.

Succinic dehydrogenase activity was inversely related to the time of HPO exposure (Fig. 1). If this were a result of proteolysis, the time of decrease in succinic dehydrogenase activity would coincide with the time of increased acid soluble nitrogen, *i.e.*, a decrease in succinic dehydrogenase activity would not occur until between 60 and 90 minutes of HPO exposure. HPO oxidation of SH groups leading to enzyme inhibition would result in a continuous decrease in activity as a function of time of exposure. The results (Fig. 1) show that succinic dehydrogenase activity decreased continuously as a function of time of HPO exposure throughout the 90 minutes. Thus the decreased succinic dehydrogenase activity is attributed to HPO oxidation of SH groups resulting in inhibition of enzyme activity, rather than proteolysis by increased free lysosomal enzymes.

Changes in free ribonuclease activities with time of HPO exposure (Fig. 1) were considerably lower than the changes in free cathepsin activity during the same time interval (Fig. 2). This could be due to differences in localization in the lysosome (membrane *vs* internal) or a reflection of differences in permeability of the lysosomal membrane to the 2 different enzymes. Changes in free ribonuclease activity show an inverse relationship with ATP concentration (↑ at 30 minutes, approach normal at 60 minutes, ↑↑ at 90 minutes) (Table I and Fig. 1). This suggests that lysosomal membrane integrity, with respect to ribonuclease permeability, is dependent on ATP.

The earliest indication of HPO damage was seen in decreased tissue ATP concentration, increased free ribonuclease activity in liver and kidney, and increased free cathepsin activity at 30 minutes of HPO exposure. Between 30 and 60 minutes ATP levels returned to normal. Free ribonuclease activity levels returned toward normal at 60 minutes, but succinic dehydrogenase activity decreased and free cathepsin activity increased with respect to the levels observed after 30 minutes of HPO exposure. During the 60 to 90 minute interval of the HPO exposure, the animals showed symptoms of acute oxygen toxicity. During this interval there was a marked decrease in ATP concentration, and a marked increase in acid soluble nitrogen and free ribonuclease activity. Succinic dehydrogenase activity continued to decrease in the 3 tissues. Free cathepsin activity continued to increase in brain and liver, but decreased in kidney. (The decrease in kidney, and only slight increase in liver free cathepsin activity is a reflection of both total and free cathepsin activity being significantly reduced at the 90 minute point.) Thus the striking changes which occured during the 60 to 90 minute interval, when the animals exhibited acute oxygen toxicity symptoms, were decreases in tissue ATP (45 to 63%), marked increases in acid soluble nitrogen (383 to 490% of controls) indicating extensive proteolysis, and significant increases in free ribonuclease activity (136 to 200% of normal) in the 3 tissues. The one change not seen until the 60 to 90 minute HPO exposure period was the marked increase in acid soluble nitrogen in the 3 tissues. This is considered to be the initial indication that irreversible damage has resulted from the HPO exposure.

Summary. Fasted male Sprague-Dawley rats (150-200 g) were exposed to 100% oxygen at 5 ATA pressure for 30, 60, and 90 minutes, and ATP concentration, succinic dehydrogenase activity, % free cathepsin activity, % free ribonuclease activity, acid soluble and total nitrogen, and tissue buffer capacity were determined on brain, liver and kidney. ATP concentrations decreased at 30 minutes, returned toward normal at 60 minutes, and showed marked decrease at 90 minutes. Free ribonuclease activity showed an inverse relationship to ATP concentration. This would implicate ATP in the maintenance of lyso-

some membrane integrity with respect to ribonuclease permeability. Succinic dehydrogenase activity decreased continuously with time of HPO exposure. Free cathepsin activity increased continuously through the 90 minute exposure in brain and liver. Free cathepsin activity increased in kidney through 60 minutes, but at 90 minutes showed a decrease which is attributed to a marked decrease in both free and total cathepsin activity at this time. Tissue buffer capacity in all 3 tissues showed a decrease in tissue acidity at 30, 60, and 90 minutes of HPO exposure. This is attributed to hyperventilation by the animal during the HPO exposures. Acid soluble nitrogen was normal at 30 minutes, slightly decreased at 60 minutes and markedly increased at 90 minutes in the 3 tissues. This marked increase during the 60 to 90 minute exposure period coincides with the time interval in which the animals exhibited symptoms of acute hyperoxia toxicity. Significance and implication of the results are discussed.

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