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SECURITY CLASSIFICATION OF THIS PAGE (When Date Ent **REPORT DOCUMENTATION PAGE** EFORE COMPLETING REPORT NUMBER . GOVT ACCESSION NO. T'S CATALOG NUM 00 **Final Report** TITLE (and Subtitle) S. TYPE OF REPORT & PERIOD COVERED Morphologic and Biochemical Effects of Oxygen Final Report April 1. 1968 - Nov. 30, 1974 Toxicity. HING ORG. REPORT NUMBER AD A 0 5 6 CONTRACT OR GRANT NUMBER AUTHOR Robert M. Rosenbaum. Ph. D. and N00014-75-2234 Murray Wittner, M. D., Ph. D. FORMING ORGANIZATION NAME AND ADDRESS AREA & WORK UNIT NUMBERS Dept. of Pathology, Albert Einstein Gollege of Work Unit 201-008 Medicine, 1200 Morris Park Ave., Bronz. N.Y. 1046 11. CONTROLLING OFFICE NAME AND ADDRESS 2. REPORT DATE Office of Naval Research Code 441 March 1, 1978 NUMBER OF PAGES Arlington, Virginia 22217 MONITORING AGENCY NAME & ADDRESS SECURITY CLASS. (of this re unclassified DECLASSIFICATION/DOWNGRADING 14. DISTRIBUTION STATEMENT (of this Report) This document has been approved for public release and salo; its distribution is unlimited. AD NU-RUD DISTRIBUTION STATEMENT (of the abstrast entered in Block 20, If different from Report) JUL 28 1978 ADY 68 SUPPLEMENTARY NOTES 1. KEY WORDS (Continue on reverse olde if necessary and identify by block number) Oxygen toxicity, cells, autolysis, lung, mitochondria, lamellar bodies, cell culture, RNA, DNA, protein synthesis, surfactant, cathepsins, histochemistry. 02 20. ABSTRACT (Continue on reverse side if necessary and identify by block number) \geq Morphologic and biochemical studies dealing with effects of high (pO_{p}) concentrations were made on a wide range of cell types including protozoa, mammalian cell lines, lung cells and marine invertebrate and amphibian eggs. Studies were aimed at evaluating effects of O, on proteclysis of cells, on mitoses, on DNA, RNA and protein synthesis, on the etiology of alveolar lining cell injury, and on the development of Q2tolerance in rats Peripheral areas related to these studies dealt with the DD 1 1473 1473 EDITION OF I NOV 68 IS OF GALLIFIC ME S/N 0102-LF-014-6601 410777

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MORPHOLOGIC AND BIOCHEMICAL EFFECTS OF OXYGEN TOXICITY

Robert M. Rosenbaum, Ph. D. and Murray Wittner, M. D., Ph. D.

Department of Pathology Albert Einstein College of Medicine Bronx, New York 10461

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Final Report April, 1968 to November 1974 Contract No. N00014-75-C-0223

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The goals and objectives of the research summarized in this Final Report are within the framework outlined for ONR contract N00014-75-C - \checkmark 0223, NR 201-008. From its initiation, these were broadly stated to be:

- a) to determine effects of atmospheric and hyperbaric O₂ concentration on living cell systems and,
- b) to relate resulting biochemical and morphologic findings to problems of oxygen toxicity.

While an immediate and pragmatic approach to understanding the etiology of O_2 toxicity would seem to require use of the cells and tissue of higher mammals, it was early decided to explore a wide range of lower organisms as regards their response to oxygen. For example, protozoons or eggs of the sea urchin or sand dollar, provide ideal material with which to investigate mitoses, intracellular digestion which itself is dependent on activation of intracellular proteases and cell death, all with respect to how or to what degree these may be affected by high O_2 concentrations. Therefore, we employed this seemingly specialized material as initial objects that could subsequently serve as models prior to actual related studies with mammalian cells. Parallel with these types of experiments we initated an ongoing effort aimed at clarifying aspects of the nature of the pathology of acute O_2 poisoning in mammals.

In the sections to follow, we summarize the basic information of all aspects of our work on this contract. Data illustrating main points or conclusions are

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from pertient publications dealing with work originating from this laboratory supported by this contract.

1. Effects of variations in methods of sacrifice on the structure of mammalian lungs.

This study was begun to clarify the liklihood that superimposed changes could be introduced in mammalian lungs exposed to O_2 , by the nature of the method of sacrifice. Such trauma-induced alterations in lung histology could confuse certain tissue alterations that might also be described as being due to pulmonary O_2 toxicity. Examples of these are atelectasis, capillary rupture, mild to severe capillary or larger vessel conjection, and alveolar wall rupture.

Rats (Carworth, males, 260 - 300 gms) were sacrificed in a variety of ways and both with and without Nembutal (ip) or ether (inhalation) anesthetic. Methods used included decapitation, ligation of pulmonary trunk, exsanguination, air embolism, blow on the head, opening of the thoracic cage, anethesia to death, both inhalation and with Nembutal. Sections of lung from animals subject to each of these procedures were fixed in 10% neutral buffered formalin, stained with hematoxylin and eosin and examined for variations in lung pathology as a result of the method selected for inducing death. No base-line controls were utilized but lung sections from animals were compared with each other for the nature and extent of each lesion, and its similarity or not to an O_2 -induced lesion.

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We could detect no differences induced by use of either Nembutal ore ther anesthesia provided that the anesthetic itself was not used to induce death. Depending on whether the respiratory center was depressed early or not, animals so killed tended to show more atelectasis, due to resorption of circulating oxygen.

Traumatic means of sacrifice should be strictly avoided when performing pulmonary O_2 toxicity studies that include or require lung histology. Even decapitation with exsanguination may involve moderate chest crush injury or abrupt, spasmodic inhalation with frequent occlusion of the primary airway, all affecting adversely the final histologic picture. Air embolism (from 5 - 10cc) was an effective way of reducing traumatic injury as was bilateral ligation of the carotids. Both these methods may be recommended.

The above considerations concerning sacrifice of animals exposed to high oxygen concentrations should be under consideration in the case of all studies involving light microscopy. Supervening changes in the appearance of lungs exposed to oxygen with animals killed by trauma can cause significant confusion in the overall assessment of the nature and extent of damage due to O_2 per se.

2. Effects of O, hyperbaric concentrations on cells.

a) Eggs of marine invertebrates.

Initial studies in the area concentrated on several pilot experiments which we intended to utilize for examination and study of mammalian cells in vitro using the light microscope. For this study, it was first necessary to design and construct an optical-pressure vessel, suitable for use with light microscopy and phase contrast accessories that, at the same time, would be able to sustain in

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excess of 7 - 10 atmospheres of pressure while incorporating satisfactory safety factors. Construction of this chamber would not only have to conform to specific optical criteria but also permit suitable temperature conditions for development of eggs in salt water.

This requirement led to construction of the optical pressure vessel shown in Fig. 1. This unit consists of two basic parts: 1) an optically aligned chamber composed of glass and Teflon sealing and spacing rings with a glass reservoir and 2) a brass sealing cap and base, the latter with internal flow channels for maintenance of constant temperature by water circulation. A major problem with both design and construction of this vessel was the question of safety since the entire chamber had to be maintained on the stage of a microscope with sufficient internal working distance and external dimensions to allow the operators to have their faces poised directly over a pressurized vessel for extended times.

Use of this chamber began with a study of effects of high O_2 concentrations on cleavage in eggs of the sand dollar <u>Echinarachnius parma</u>.

Mature specimens of Echinarachnius parma were collected at the U.S. Fish and Wildlife Service, Boothbay Harbour Laboratory. Gametes were obtained by use of electrodes applying 10 volts about the aboral surface. About 0.2 - 0.4 ml of so-called "dry" sperm was diluted with seawater (1:1). Several drops of this preparation were then applied to washed eggs so that fertilization could proceed at 20° C. Fertilized eggs were placed in the optical chamber and examined by phase contrast for signs of subsequent cleavages against a time base. Gas mixtures consisted of 100% or 99.9% oxygen as the experimental mixture to the desired pressure and 3 psi O₂ and nitrogen to the indicated

-4-





Fig. 2

Fig. 1 — Cross-sectional and upper surface view of the optical-pressure vessel employed in these experiments. The components a, b, c, c' and d are not drawn to scale.

Fig. 2 —Time required to reach fifty per cent first cleavage in eggs continuously exposed to various pressures of pure oxygen and to oxygen-nitrogen control mixtures for 4 hours. Development at room temperature (20°C). C - C, 99.9 per cent oxygen; $\bullet - \bullet$, 3 psi oxygen – nitrogen to indicated pressure.

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guage pressure. Gas exposure in each case was produced at least in duplicate at pressures of 15, 30, 60, 78 and 120 psi (abs.). The time required to reach 50% first cleavage under the equivalent of a single atmosphere O_2 + nitrogen to equal the desired experimental pressure was approximately 100 minutes at the pressures employed. With use of pure O_2 , first cleavage was reached in 50% of the eggs by 100 minutes at 30 psi, by 50 - 60 min. at 60 psi and by 180 - 190 minutes at 112 psi (Fig. 2). These results gave the first indication that increasing pressures of O_2 had a direct effect on the initiation of first cleavage in the sand dollar egg. During these observations, direct use of the light microscope permitted assurance that no abnormalities were produced in eggs under pressure.

It was next desired to test whether high O_2 pressure which we know could have effects on the rate of early cleavage in sand dollar eggs also produced abnormal subsequent cleavages. At pressure of 15 to 45 psi (aba) O_2 , no effect on development could be detected and the 32 cell stage was reached in approximately 240 minutes. At 60 psi, some acceleration in cleavage over normal air controls was reached. Marked inhibition of cleavage took place with 110 - 115 psi O_2 and these eggs did not undergo first cleavage until about 180 minutes at which time normal air controls were already in third cleavage. When eggs from this series of experiments were returned to normal air, all but those eggs exposed to 110 - 115 psi O_2 developed normally. Controls exposed to 1 atm (aba) $O_2 + N_2$ to equal 110 - 115 psi total guage pressure, developed normally when returned to air. These experiments indicated that at

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a specific pressure, O_2 can induce inhibitory effects on cleavage in the sand dollar egg and that this effect is due primarily to high pO_2 concentrations, not pressure <u>per se</u>. Spindle visualization <u>in situ</u> in those eggs arrested at the 2-cell stage with 110 - 115 psi O_2 for 4 hours was attempted using long working distance optics and a Wratten 88A filter with Kodak infra-red film. These photomicrographs clearly showed the outline of an intact achromatic figure including two astral figures with interconnecting spindle.

Short exposure to high O2 concentrations and its effect on the time required for 1st cleavage was also studied. Variations in these short exposure times were deemed worthy of study because of the possibility that different amounts of endogeneous SH complexes or cofactors that could govern resistance or susceptibility to O, might be present. Five minutes following fertilization as evidenced by the presence of elevation of the vitelline membrane, batches of 15 - 20 eggs were placed under 35 - 45 psi, 60 - 70 psi and 110 - 115 psi O2. In addition, normal air controls and controls exposed to 3 psi O_2 + 112 psi N_2 were also employed. Gas exposure was applied only for 30 or 60 minutes. Below O, pressures of 110 - 115 psi, there appeared to be no effect on the time required for onset of 1st cleavage. With pressures of 110 - 115 psi, there were abnormalities both with respect to the time required to reach first cleavage and in the stage of development attained compared to air and nitrogen pressure controls. These experiments demonstrated a cumulative effect of high O, concentrations. The longer the exposure to a critical concentration of oxygen, the greater the difficulty in overcoming inhibiting effects of the gas as expressed by a delay in initiation of 1st cleavage.

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These early studies pointed to alterations of sol/gel states as a possible responsible factor in the formation and breakdown of the mitotic spindle and probably other cytoplasmic factors as well. Some effects described on the basis of our studies can be taken as temporary but direct O_2 blocking effects on mitotic spindles of those cells studied ware apparent. Also a reversal of this effect was obtained upon return to normal air.

b) Amphibian eggs

When frog (Rana pipiens) eggs are exposed to high (3 - 6 atm. abs) O, pressures immediately following fertilization as visualized by vitelline membrane elevation, development proceeds to the gastrula stage. At the time of initiation of gastrulation, the development of the O₂-exposed egg is blocked. Such blocked eggs do not develop further, even if returned to normal air but if the blocked gastrulae are maintained under O, pressure of 3 atm. abs. +, they do not cytolyze. By way of contrast, blocked eggs returned to normal air do cytolyze. These observations suggested a means for demonstrating control of autolysis and proteolytic activity by high pO, concentrations. Since a number of proteolytic enzymes are dependent on SH groups as cofactors, we wished to measure the levels of such catheptic activity in O2-blocked/unblocked gastrulae. Using purified casein as a standard substrate, we determined enzyme levels representing non-specific cathepsin activity at 725 mu. Values for catheptic activity were obtained for normal development of R. pipiens through to tadpole feeding stages. There was a gradual rise in enzymic activity beginning at late cleavage and continuing on past the tail bud stage. By utilizing O, high

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pressures with single atmospheric concentrations of O_2 + nitrogen to make up the experimental pressure equivalents as controls, it could be shown again that pressure was not in itself a factor inducing the inhibition. For example, enzyme activity was markedly inhibited in eggs exposed to 7 atm (abs) O_2 for 24 hours in contrast to persistent normal enzyme activity in control eggs exposed to 1 atm. abs. O_2 + 6 atm. abs. N_2 (Fig. 3 a).

In order to demonstrate that the O_2 -induced gastrula block might be due to effects of SH inhibition, a series of O_2 exposures was done to demonstrate removal of the block as a result of SH protection. For this, sulfhydryl compounds were tested at various concentrations. These "protective" agents include reduced glutathione (0.01 - 0.001M); BAL (British anti-Lewisite, 0.01 - 0.001 M); AET (aminoethylisothiouranyl - Br) at concentrations of 0.01 M - 0.001 M); cystamine HCl at 0.01 - 0.001 M and mercaptoethylamine at 0.01 -0.001 M. With frog eggs exposed to 7 psi (guage) O_2 gastrulation took place with the presence of SH-reagents. Repeat of these experiments several times indicated that the gastrular block may be both prevented and overcome by suitably timed SH applications. In those eggs that became blocked, cytolysis did take place provided sufficient amounts of endogenous SH reagent wrepresent. AET and mercaptoethylamine were the most successful agents in restoring the ability of the marine eggs of invertebrates to autolyze following the gastrular block.

We next determined possible reduction or loss of catheptic activity in gastrular blocks induced by means other than O_2 . This was necessary in order to assess

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whether O_2 had a specific effect on proteolytic activity or whether a gastrular block <u>per se</u> was associated with the inhibition of enzymic activity (Fig. 3b). Gastrular block was accomplished in two ways:

1) by uncoupling of phosphorylation using 2, 4-dinitrophenol and 2) by use of supramaximal temperatures. Eggs were exposed to temperatures $(31^{\circ}C)$ for 48 hrs. at which time control eggs developing at $18^{\circ}C$ were in the medullary fold stage. The heat-treated eggs were arrested at the gastrula stage and, soon thereafter, began to cytolyze at which time they were assayed for catheptic activity. A similar effect was obtained when 2, 4 dinitrophenol (DNP) was employed for production of gastrular blockade. Maximum numbers of gastrular arrests occurred when 1:500,000 DNP was applied to <u>R. pipiens eggs at the 2-cell</u> stage. Subsequent experiments employed 1:1,000,000 DNP which was less toxic and caused approximately 80% of the eggs to become arrested at the gastrular stage. In both the cases, gastrular block by temperature on the one hand and DNP on the other, catheptic activity rose sharply as arrested eggs underwent disintegration.

The above series of experiments clearly showed that among the three procedures used to generate a gastrular block (high O_2 concentration, dinitrophenol and supramaximal temperatures), only oxygen was able to inhibit subsequent cytolysis of the arrested eggs. This inhibition of cytolysis was accompanied by an inhibition of proteolytic activity as marked by a decrease in cathepsins.

3. Catheptic activity in protozoa

The dependence of protozoa on activity of intracellular hydrolases for digestion of food was studied with a view to more closely defining an earlier

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series of observations made in our laboratory. During experiments concerned with using <u>Paramecium</u> ciliary beat as an endpoint for lethal effects of O_2 toxicity on cells, it was noted that even when cells died as indicated by total loss of ciliary beat, the dead cells did not cytolyze under oxygen. Indeed, when dead Paramicium remained under oxygen pressures of 3 - 7atm. (abs.) for several weeks, the cells maintained their structural integrity and did not cytolyze.

This pnenomenon was investigated using a single line clone of <u>Paramecium</u> <u>caudatum</u> established according to the method of Sonneborn (J. Exp. Zool. 113, 87, 1950). Aliquots from these cultures consisting of 16,000 animals were exposed to oxygen in a lucite and brass pressure vessel. Decompression as well as recompression was extended over a 10 minute period to avoid physical damage to the cells. Since temperature is a known factor mediating various levels of O_2 toxicity, all temperatures were maintained at $26^{\circ}C$. Three pressures used consisted of 3 atm. (abs.) and 7 atm. (abs.) of pure O_2 and a single atmosphere of air with the addition of 6 atm. (guage pressure) of pure N_2 as a control. Paramecia exposed to 3 atm. O_2 were killed by 9 hr. whereas those exposed to 7 atm, were killed by 3 hours as measured by loss of ciliary beat and cessation of the pulsatile motion of the contractile vacuole. Control animals remained alive throughout these exposure times. For these experiments, some dishes with dead animals were maintained under O_2 for up to three weeks, other dishes were returned to normal air following decompression.

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Proteolytic activity was determined in homogenates of approximately 16,000 cells using hemoglobin as substrate. These assays showed that, with both the pressures of pure O_2 employed (3 and 7 atm. abs.), proteolytic activity was inhibited. In contrast, organisms killed by heat shock ($60^{\circ}C/15$ sec.) only showed persistence of proteolytic activity (Fig. 4,5). Further, exposure of organisms to 10, 45 and 60 minutes respectively of pure O_2 demonstrated decreasing proteolytic activity in relation to longer exposure times to O_2 (Fig. 5) and emphasized the inhibitory role of O_2 .

These experiments suggested two distinct phenomena to explain the effects of O_2 at the pressures employed. One is the direct O_2 -inhibition of SH-dependent proteolytic enzymes resulting in reduction or total inhibition of protein break-down in dead cells. However, a second factor to consider is the alternate possibility that, at the pressures used, O_2 had a stabilizing effect on the membrane-bound lysosomes. With this concept, alterations in the lysosome membrane could "fix" or stabilize it against permeability to endogenous enzyme protein and so prevent release of membrane-bound hydrolases, including non-specific cathepsins. Although such basic structural changes in lipid membranes might be brought about by lipid peroxidations, we must emphasize that this has not been demonstrated in the case of lysosomal membranes.

The latter concept next led to a study of relationships between food or digestive vacuoles in <u>Paramecium</u> and the so-called neutral red bodies, the primary lysosomes of this ciliate. In addition, since we were also interested in possibly demonstrating the O_2 -inhibition of enzyme release from stabilized lysosomes using cytochemical methods, we began to explore a range of cell systems for convenience in studying these problems.

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4. Cytochemical studies on lysosomes

We first compared two distinctly different light microscopic histochemical methods for visualizing acid phosphatase activity since this class of hydrolase has been frequently regarded as a marker for lysosomal activity. Utilizing a lead salt method with β -glycerophosphate as substrate on the one hand and an azo dye method with naphthol phosphate as substrate on the other, we were able to demonstrate that the two methods could reveal different acid phosphatases. Further, we were able to demonstrate a significant species variability in the two substrate defined enzyme classes. A high degree of selectivity for visualizing acid phosphatase activity by each method could be demonstrated clearly by using a method of sequence staining. For this, one substrate technique would be applied to a given tissue section followed by incubation using the second substrate resulting in three classes of staining - a) staining by the lead method only; b) staining by the azo dye method only and c) staining by both methods superimposed. We dealt with this type of study in depth, applying specific inhibitors for each visualization reaction for a different acid phosphatase as well as a variety of different naphthol substrates. Of these, naphthol AS-BI provided the best intracellular localization for an azo dye method while Na-β-glycerophosphate persisted in being the best method for metal salt type reactions.

Our aim, following these studies was to demonstrate resistance or susceptibility to high pO_2 levels, on the part of lysosomes from a variety of cells. Our rationale was based on the above described series of observations with

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paramecium, marine invertebrate eggs and frog eggs that pointed to a loss of proteolytic activity under high pO, levels.

One test object that could provide ideal material for such histochemical studies at the light microscope level was the digestive gland of the snail, <u>Heix pomatia</u>. This small land snail could readily be exposed to O_2 and containes literally a "sack" of digestive enzymes in a gland complex located in a series of gut diverticula. This digestive complex is readily accessable to O_2 and contains a cell type - the secretory - resorption cell - which contains a readily defined lysosome complex in the form of colorless granules. These granules showed intense staining for several acid hydrolases including β -glycuronidase, and intracellular peptidase activity of several different types. The metal sait method for acid phosphatase activity could not be used in studying the gland complex due to the presence of large numbers of metal-salt binding calcium granules throughout which yield false-positive staining reactions. Azo dye methods for acid phosphatase activity, on the other hand, showed positive reactions in the gland complex. none of which became localized in the secretory absorption cells.

<u>Helix pomatia</u> was exposed to high O_2 concentrations as follows: 36 snails were stained for 10 days. The animals were divided into 4 groups, 18 animals served as controls and were further divided so that 9 provided normal air controls and 9 were exposed to an atmosphere of air on which was superimposed 6 atm. (guage) of N₂ (7 atm. abs.). The experimental animals were exposed to O_2 in 2 groups; 9 animals to 3 atm. abs. O_2 , the remaining 9 to 7 atm. abs. O_2 .

During the experiments which lasted for 10 days, all animals were given food ad libitum. With food once again available, all snails began feeding within minutes. They were monitored at least twice daily during which time feeding was observed. At the conclusion of the experiment, animals were prevented from further feeding while still under pressure and all animals from all four groups were weighed. All control animals had gained weight which, when compared to experimental animals, was approximately 3 - 4 times the experimental weights. We also consistently monitored the aquaria of both control and experimental groups and could detect the absence of fecal strings in animals exposed to O₂. Concentrations of 7 atm. O₂ had a lethal effect on the majority of snails in this group (5 out of the 9 snails were dead). Autopsy of all living O₂-exposed animals provided additional evidence that O₂ at both 3 and 7 atmospheres did not allow digestion in these snails. Whereas control animals had evaculated or digested gut contents during the post-feeding starvation period, the gut of O₂-exposed animals was full of food and showed no sign of digestion. The guts of all animals were removed and analyzed histochemically for detection of hydrolytic enzyme activity. The secretory-resorption cells of the digestive gland showed normal levels of enzymic activity whereas there was reduced levels of β -glucuronidase and proteolytic esterases in the SR glands of Og-exposed animals. We could not determine whether exposure to O, had any effect on the primary lysosomes represented by the so-called "clear" vesicles in the SR cells.

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In order to determine whether O_2 had a direct effect on primary lysosomes, we next studied the neutral red granules of paramecium. It has long been known that application of the vital dye neutral red reveals a cortical distribution of stained vesicles. Subsequent feeding, with the formation of food vacuoles, results in rapid migration of the neutral red stained cortical granules to the rim of the forming food vacuoles.

We undertook a series of histochemical observations to reveal whether movement of the neutral red cortical granules to the food vacuoles could be associated with feeding, food vacuole formation and subsequent intracellular digestion in our hands. Using activity for acid phosphatase and several intracellular proteases as a marker, this relationship was indeed established. A next step in this series of experiments dealt with exposing paramecium to high O_2 concentrations with the rationale that this would limit release of intracellular proteolytic activity, either by producing a block to the migration of neutral red stained primary lysosomes or by limiting release of enzymes once the lysosomes had made contact with the food vacuole. The latter would involve stabilization of the lysosomal membrane. These experiments could not be carried out successfully since complete digestion within the paramecium food vacuole requires an excess of 6 hours and our organisms succumbed to oxygen toxicity within 3 hours at the range of temperatures $(12 - 18^{\circ}C)$ or pressures (3 - 7 atm. abs.) used as measured by cessation of ciliary beat.

5. Protease activity in vertebrate lungs

Further studies on effects of oxygen on proteolytic enzyme activity that we undertook employed vertebrate lungs. Rats of the Holtzman strain (250 - 300 gms)

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were exposed to 100% O_2 at 760 mm Hg for various times up to 60 hours. O_2 concentrations and CO_2 levels were monitored continuously by a Beckman Polarographic O_2 analyzer or a Barber-Coleman Gas Chromatograph (see Fig. 6). Two kinds of biochemical determinations were undertaken; the first was to indicate changes in amounts of protein in a constant mass of lung tissue, the second was an assay for endogenous cathepsin activity in homogenates of O_2 exposed and normal air control rat lungs.

Levels of endogenous catheptic activity in normal air lungs showed that 95 -98% hydrolysis of a hemoglobin substrate was achieved by 8 hours. Frequently, maximal hydrolysis would be achieved by 6 hours. The possibility that some of this activity might be due to bacterial action was ruled out by using aseptic techniques especially for removal of lung tissue. Excised rat lung fragments exposed to 4 ata O₂ showed a abrupt decline in the activity of lung tissue towards a hemoglobin substrate. The possibility that pressure itself might be a factor in the O₂-induced decrease in catheptic activity was tested using exposure to 14 psi air + 105 psi No. No inhibition of cytolysis was obtained nor could it be induced by thermal shock at normal atmospheric pressures. The effects of time on Og-induced inhibition of catheptic activity were also studied using constant (120 psi) exposure pressures to O2 and varying the duration of each exposure. It was possible here to demonstrate a direct relationship between duration of exposure at a constant pressure and the number of hours required for a constant reduction in the rate of enzyme hydrolysis. Enzymic activity returned upon the return of homogenate to air.

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Fig. 6 Schematic of the apparatus used for exposure of fats to prolonged periods of adaptation, as well as subsequently to toxic levels of O_a treatment at single atmospheric pressures.

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With 10 and 20 minute exposures, enzymic activity returned rapidly in the case of lung homogenates while with a 45 minute exposure to 120 psi O_2 , partial recovery took place. This latter observation suggested that higher pO_2 concentrations are able to induce irreversible recovery.

Because many intracellular proteases are known to contain SH cofactors, we wished to also assay the total lung tissue SH and its stability using in vitro exposures in normal air and under hyperbaric conditions. The data generated from these assays stated that the decline in tissue SH as a result of O_2 exposure was directly responsible for weak enzymic reactivity towards hemoglobin substrate. Once it was possible to relate the presence or absence of tissue SH to strength of proteolytic activity, a second series of experiments were undertaken to see if exogenous SH reagents could induce a protective effect on proteolytic activity (Fig. 7).

We could also demonstrate that peptide bonds accumulating in the alveoli of rat lungs were indirectly the primary early targets for O_2 toxicity. Experimental material for this study was produced by first exposing rats to $100\% O_2$ (at 1 atm. O_2) and excising their lungs by 36 - 40 hours at which time they show signs of so-called O_2 -pneumonia. Lungs of such animals become filled with a proteinaceous exudate and aliquots of homogenates from this material were assayed quantitatively for the amount of peptide bonds present using lungs from normal air exposed rats as a control. Within 24 hours from the time of initial exposure to $100\% O_2$, there was a significant rise in peptide bonds present in the lung homogenate samples (Fig. 8). A second rise in peptide bonds

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FIGURE 7 Recovery effect of SH-protective compounds on catheptic activity added after exposure of lung homogenate from a normal rat to 120 psi O₂ for 45 min. Samples of protozoa homogenate exposed to identical O₂ concentrations with SH-protective reagents showed no increase in enzymic activity

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took place at 40 hours. These sequential increases in peptide bonds are possibly attributed to two phenomena - the first, related to the accumulation of exudative protein in alveoli of O_2 exposed lungs, the second, due to failure of the normal proteolytic mechanism that would be at play in normal lungs not exposed to O_2 . Resistant catheptic levels in normal air control lungs and in lungs exposed to high pO_2 levels for 4 hours with return to air and a subsequent 4 hour O_2 exposure daily for 10 days resulted in a significant reduction in peptide bonds and higher levels of proteolytic activity than when continuous O_2 exposure beyond 4 hours was employed.

The above studies appear to have been the first dealing with effects of high pO_2 levels on proteolytic enzymes of lung and especially on the O_2 -induced exudative of lung. They suggest that the proteinaceous pulmonary fluids induced by O_2 toxicity may not be cleared up because of an O_2 -induced inhibition of proteolysis possibly due to SH cofactors blocked as a result of O_2 exposure. Although we made many attempts to reveal lysosomes associated with alveolar macrophages under these conditions and the alveolar lining cells themselves, we could not employ satisfactory histochemical methods that were suitable for observation with other than the transmission electron microscope.

6. Studies with mammalian cell cultures

Our early studies with a wide range of single cell systems were designed to test what might be required for developing a system capable of testing the response of <u>in vitro</u> mammalian cells to HPO. When exposing cell and tissue cultures

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to gases under pressure, several technical problems must be solved. At the time we began these studies, to our knowledge, no tissue cultue system had been exposed to high pO_2 levels. The vessel required for such a study, must have the following qualifications: 1) it must permit continuous observation of the cells while under pressure; 2) sterility must be maintained and 3) the entire system must withstand the required pressures and also must be able to confine a gas with the high fugasity known to be a property of oxygen. In addition, since nearly all culture cells we studied died rapidly at high pO_2 levels above 3 atm. abs., the cultures used should be well established cell lines whose dynamics should be fully understood. Finally, a basis for recognizing O_2 -induced cytopathic effects must be developed for such <u>in</u> <u>vitro</u> systems.

Of the several culturing methods tried, the apparatus diagrammed (Fig. 9) was used in various versions. With long-working distance phase contrast optics, it became possible to make fairly precise determinations of some aspects of cell injury within this <u>in vitro</u> system. The cells used as test objects for these studies were from HeLa, HLM, L5178T and Y5 strains. None were differentiated cell lines and most of the sublines employed had lost contact inhibition. The initial effect of exposing this material to O_2 at pressures above atm. abs. appeared to be a limiting of both anaerobic and aerobic O_2 utilization. In any case, no cell culture was ever able to survive beyond 12 hours in 100% O_2 . A series of experiments were done to test the limits of such disruption in O_2 utilization in a

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population of mouse L-cells. In order to measure the effects of O_2 on respiration, an in vitro formazan assay with an excess of succinate in the medium as substrate, was introduced. In these experiments, a population of L-cells were exposed to 95% O2 or 2 atm. O2 for a maximum of 12 hours to establish irreversible decline of the culture under these conditions. A second culture of L-cells was run simultaneous using only 95% O2 with a tetrazolium marker indicating the ability of the cells to still engage in hydrogen transfer. At intervals of two days, subcultures of these O2 exposed cells were returned to air. As each culture was returned to normal O2 concentrations, formazan formation as the result of increased oxidative metabolism could be seen. These series of experiments signified that, at threshold O2 concentrations, the cultured cells were still able to maintain a metabolic reserve permitting O utilization with hydrogen transfer upon return to normal air. With higher O, concentrations (2.5 - 3 atm. abs. O2) complete breakdown of cell cultures occured by 12 hours. Such irreversible damage was heralded by release of cells from their substrate, the rapidity of which was, in part, able to be controlled by increase in the pO, levels up to 3 atm. abs.

Using synchronized HeLa (s) cells grown in spinner cultures, we have determined that the S phase of the cell cycle appears as the most labile to O_2 while the post-synthetic G_2 phase appears the least sensitive as judged by the viability studies outlined above. DNA itself does not appear to be, affected by O_2 (see below).

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We have also attempted to extend these cell studies in part by using explants of fetal and adult lung. Our hope here was to take advantage of cells that might migrate out of such larger explant fragments exclusive of fibroblasts. To this end, initial experiments did indeed show a range of cell types migrating out of lung explants. These included fibroblasts, macrophages and a variety of epithelial types which could not be characterized further. Failure to and inability to further characterize these cells and the heterogenous nature of the population led to our abandonment of the project.

7. Effect of oxygen on intranuclear RNA synthesis

The effect of hyperatmospheric O_2 on macromolecular synthesis of intranuclear RNA was studied in HeLa cells. The investigations were carried out to ascertain whether O_2 toxicity specifically interfered with cell synthesis of protein, RNA and DNA.

Initial studies revealed that at 720 mm Hg O_2 and 40 mm Hg pCO_2 , exponental growth of the cell population was depressed. This became evident after the second generation or doubling time of the growing cell population. RNA, DNA and protein synthesis were next studied in cells exposed to O_2 at 1480 mm Hg (Fig. 10). During the period studied, i.e. 60 minutes, partial inhibition of RNA synthesis only was detected while DNA and protein synthesis seemed unaffected for this time period, although if the period of exposure to O_2 was extended partial inhibition of protein synthesis could also be shown.

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Further studies were carried out in order to determine the manner by which hyperbaric O_2 inhibited RNA synthesis. Intranuclear RNA synthesis was studied after exposing cells to 1480 mm Hg O_2 by uridine ¹⁴C pulse labelling techniques. The cells were ruptured and separated into nuclear and cytoplasmic fractions. The former was further subfractionated into nucleolar and nucleoplasmic portions. It was demonstrated by this method that the normal processing of nucleolar RNA from 455 to 285 was blocked by hyper-atmospheric O_2 although cleavage to the 185 moiety seemed to proceed unabated. In these studies, we were able to distinguish a complete block to ribosomal RNA processing at a time when DNA and protein synthesis were still unaffected by elevated pO_2 tensions. The precise block in rRNA processing appears to be in the 455 cleavage reaction that occurs in the nucleolus such that neither 325 or 285 rRNA moieties are formed or transported to the cytoplasm under elevated pO_2 levels (Figs. 11, 12, 13).

As a result of these studies, one could expect that the disruption of rRNA synthesis would quickly be followed by decreased protein synthesis; DNA synthesis, which is dependent upon intact protein synthetic mechanisms in turn would be inhibited. These studies have provided support for our morphological observations of early disruption of rough endoplasmic reticulum of lung cell types by elevated pO_{o} .

8. Ultrastructural studies on mammalian lung cells exposed to oxygen.

Our interest in the response of a wide range of cell types to oxygen toxicity and our demonstration that there are defects in oxidative metabolism in both



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non-mammalian and mammalian cells exposed to high pO_2 concentrations, led to ultrastructural studies outlined in this section.

During the course of exposing rats to 1 atm. concentrations of pure O2, we noted that, while the majority of adult animals underwent pulmonary O, toxicity by 60 - 72 continuous exposures, a certain percentage of animals did not show signs of pulmonary edema. During the course of exposing rats to a range of different O2 concentrations, we further noted that animals exposed to the lower concentrations (below 100% $O_2/760$ mm Hg) did not die and, furthermore, when these animals were subsequently placed in lethal O_2 concentrations (100% atm. abs./760 mm Hg), they survived for many weeks. Such animals were first referred to as "O2-adapted" but subsequently were called "O2-tolerant". Further experiments with such O2-tolerant rats indicated that, once exposed to lethal O_2 concentrations, they could be returned to normal air and so proceed to an apparent normal life span. It appeared, therefore, that O2-tolerant rats underwent the best protection against pulmonary O, toxicity at normobaric pressures yet devised. Full examination of the lungs of Og-tolerant rats revealed no basis for judging that there were significant changes in these lungs at the light microscope level. Tolerant animals exposed subsequently to 100% O, for up to 6 weeks also showed no sign of pulmonary O_2 toxicity with the light microscope. Accordingly, a systematic electron microscope study was undertaken in order to seek the cellular basis for tolerance and to determine whether there was any ultrastructural damage.

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Rats were systematically exposed to tolerance-inducing levels of O_2 (85%) and to lethal O_2 levels (100%) for various times. Some animals were exposed in sequence, others were maintained at one concentration of O_2 throughout the experiments. Lungs were fixed in glutaraldehyde (3.5% in cacodylate buffer, 0.08 M at pH 7.2) and post-fixed in 1% OsO₄.

Ultrastructural examination of these tissues showed especially clear cut changes in type 2 alveolar lining cells of all O_2 -tolerant animals. These changes included especially marked mitochondrial hypertrophy and highly unusual "tea cup" or stacked, cup-shaped mitochondrial forms. This conformational change is in sharp contrast to the bean-shaped mitochondria so characteristic of normal type 2 cells seen in lungs of air exposed animals. Determinations of the mitochondrial volume change on the part of O_2 -tolerant rats in contrast to normal air controls yielded a 200 - 300 percent increase in mitochondrial volume. These conformational changes persisted when O_2 -tolerant rats were subsequently exposed to 100% O_2 concentrations. Reduction in mitochondrial volume appeared to take place when these animals were returned to normal air although we have not followed up this phase of the post exposure period.

In contrast to O_2 tolerant rats, the lungs of O_2 -toxic animals showed alterations in mitochondria that could very well account for the failure in respiratory metabolism we had observed in cultured cells exposed to high pO_2 levels. Mitochondria in type 2 cells from toxic animals' lungs were swollen, showed severe disruption of cristae and had also lost matrical density suggestive of loss in the ability to phosphorylate.

The clear cut effects noted above for type 2 alveolar lining cells next led us to survey all the alveolar lining and interstitial cells in lungs of animals exposed to O, toxicity. It was our aim here to determine whether different lung cell types showed different degrees of susceptibility to lethal pO2 levels. This study was undertaken by generating a close-cut series of animal exposures to 100% O, at 2, 4, 8, 12, 14, 18, 21 and 28 days respectively using O,-tolerant animals. This was supplemented by animals exposed to 100% O, for times up to the maximum of 72 hours. As a result of these observations, a clearcut sequence in the O_2 susceptibility of the three major cell types of the air/blood barrier emerged. The most susceptible cell type was the type l or squamous epithelial cell lining the alveolae. The capillary endothelium reacted next while the type 2 epithelial cell appeared the most resistant to O2 toxicity. The cytopathic effect by O2 on each of these cells would also be defined clearly. Most typically this appeared initially as swollen mitochondria with rapid loss of matrix density. The cells themselves showed signs suggestive of loss of water balance with resulting swelling and vacuolization of the endoplasmic reticulum and generalized cellular edema. The granular ER became totally disrupted with the resultant scattering of disengaged ribosomes. Following these early to middle O2-induced cytopathic effects, type 1 cells could be seen lifting off their basement membranes.

These studies also made possible further observations on features in the resistant type 2 epithelium that could be related to the O_2 -tolerant effect. A clear-cut increase in the number of multivesicular and complex bodies could be seen in this resistant cell type. Since these cytoplasmic organelles are

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definitely associated with formation of the osmiophilic lamellar bodies, an increase in their number would be of significance in relation to alterations in secreted phospholipid on the part of the type 2 cell.

To test the possibility that O_2 -tolerance was associated with changes in synthesis of surface active phospholipids, a series of O_2 -tolerant and $O_2^$ toxic animals were examined as to the level of surface tension induced by extracts of their lungs. O_2 -tolerant rats were found to generate lower surface tensions (dynes/cm²) on a Wilhelmi surface balance in contrast to normal air controls. The lowest surface tensions we obtained in this study where taken from washings from O_2 -toxic rats.

Effects of O on other organ systems

The effects of O_2 -toxicity on organs other than lung have been infrequently studied. The investigation described in this section was undertaken to define a possible marker for O_2 -toxicity using serum from exposed animals as an enzyme source.

We began to study the enzymes involved in tryptophan metabolism in liver since at least one pathway of tryptophan metabolism, tryptophan hydrolylase, is the rate-limiting enzyme in the biosynthesis of 5-hydroxytryptamine (5-HT). 5-HT has been thought to be a factor in pulmonary O_2 toxicity. The second biosynthetic pathway for tryptophan concerns the heme-dependent enzyme, tryptophan pyrrolase, which we showed increases significantly in O_2 toxicity (Fig. 14). This increase correlates well with the synthesis of the apoenzyme



116. 14 Percent of control activity of tryptophan hydroxylase and tryptophan pyrrolase activity in livers of rats exposed to 100°; O2 (760 mm Hg) for various times. Each point represents average values from 4 to 8 animals at each exposure time.

beginning by 24 hours with $100\% O_2^{*}$. The increase in both appenryme and the enzyme per se does not appear in the O_2 -tolerant animal. This suggested to us that the increase in tryptophan pyrrolase activity, which is under control of adrenal cortico-steroids, might be useful as a marker of a stress reaction in O_2 -exposure. The rise in the levels of enzyme and appenryme, interestingly enough, occur simultaneously at the time that the most severe pulmonary damage is beginning in the O_2 -toxic animal. Extended studies using the rise in pyrrolase activity as a marker suggest that it may prove useful to mark a period of irreversibility with respect to pulmonary O_2 -toxicity.

A basic etiology for O toxicity at the cell level with physiologic inter-

These studies have led us to a range of concepts as to the role of O_2 on specific lung cells especially under concentrations deemed toxic. Our in vivo and in vitro studies have demonstrated certain enzyme inhibitions associated with exposure to high O_2 concentrations which could account for the proposed alveolar cell defects suggested in Fig. 15. These could, in turn, lead to the complex gross and microscopic pulmonary changes indicated. Such a scheme, whereby the alveolar lining cells show varying degrees of resistance and susceptibility to high pO_2 , could also seem appropriate to account for a cellular basis for O_2 -induced tolerance to otherwise lethal oxygen effects. On the basis of our studies and observations, for any protective scheme, we would stress the importance of maintaining naturally O_2 -resistant lung cells such as the type 2 epithelial

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cell lining the alveoli The next phase of research in this area will be most fruitful when fully defined mammalian lung cells are grown in tissue culture with minimal loss of differentiation and in sufficient numbers so as to provide material for meaningful biochemical studies.

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