# THE EFFECTS OF INCREASED OXYGEN TENSION ON THE CAPILLARY DEVELOPMENT OF THE CHICK CHORIOALLANTOIS

by

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## I. The Problem

To determine the effects of elevated environmental $pO_2$ on capillary development in the chick chorioallantoic membrane.

> "A moralist, at least, may say, that the air which nature has provided for us is as good as we deserve."
>
> Joseph Priestley (1)

## II. Historical Perspective and Survey of Literature

### A. Early Observations.

The first recorded observation of the biological effects of increased $pO_2$ was made in 1774 by Joseph Priestley who, following the isolation of elemental oxygen from mercuric oxide, described the effects of breathing this "dephlogisticated air" (1): "The feeling to my lungs was not sensibly different from that of common air, but I fancied my breast felt peculiarly light and easy for some time afterwards. Who can say but that in time this pure air may become a fashionable particle in luxury? Hitherto only two mice and myself have had the privilege of breathing it."

Priestley also cautioned that as a candle burns much faster in this "pure" air so might a man breathing it "live out too fast."

The first adverse effects of increased $pO_2$ were noted by Lavoisier in 1783. Repeating the experiments of Priestley, he noted an "incendiary" action of pure oxygen on the lungs of guinea pigs (2).

Extensive studies of the acute effects of increased $pO_2$ were undertaken by Paul Bert around 1873 (3). He was first (2) to recognize that the observed biological effects of increased oxygen were related to the product of oxygen concentration and the total ambient pressure; in other words, to the partial pressure of oxygen. He found increasing the ambient pressure of air ($1/5O_2$, $4/5N_2$) to 15 atmosphere pro-

duced the same results as raising the pressure of 100 percent $O_2$ to three atmospheres thus demonstrating that the observed effects were due to the elevated partial pressure of oxygen rather than to the elevated partial pressure of nitrogen or the total gas pressure. His experiments utilized a wide variety of animals including reptiles, birds and dogs and were conducted at many different partial pressures of oxygen. He observed that partial pressures of oxygen greater than three atmospheres (2280 mm. Hg.) were usually necessary to produce generalized convulsions in animals, that if this exposure was terminated at the onset of the convulsions the animals survived without noticeable defects, but if exposure and convulsions continued many of the animals demonstrated permanent neurologic damage.

Lorrain Smith, in 1899, described pulmonary changes in animals exposed to increased $pO_2$ for prolonged periods (4). He confirmed that convulsions were produced in birds at 3 atm. $pO_2$ and in mice at 4 1/2 atm. $pO_2$. He also noted that although 40 percent $O_2$ at one atmosphere of pressure produced no apparent effects, mice in 70-80 percent $O_2$ at one atmosphere became dyspneic and died within 4-8 days and mice exposed to 3 1/2 atmospheres $O_2$ died in about five hours. The lungs of all the dead mice showed congestion, consolidation and edema. Smith also noted wide individual variations within and between species in tolerance to high oxygen pressure.

During the past sixty years a large amount of research has explored the mechanisms of oxygen toxicity (5), the uses of oxygen as a therapeutic adjunct (6), and the optimal partial pressures and gas mixtures for manned sea and space explorations (7).

B. Hyperbaric Oxygen

Oxygen toxicity has been observed in virtually all forms of life including plants, bacteria, protozoa, annelids, insects, reptiles, birds and mammals (8). Many biological effects of increased $pO_2$ have been observed.

Most of the biological effects of increased environmental oxygen tensions below one atmosphere are observed to occur more rapidly at oxygen tensions above one atmosphere. Vascular and pulmonary changes, important examples of common effects, will be discussed in a later section dealing with effects of oxygen tensions of one atmosphere and below. In addition, oxygen pressures above one atmosphere (hyperbaric oxygen or HBO) produce some effects, e.g. convulsions, not observed at tensions below one atmosphere. Because much more information is available on the effects of HBO than on the effects of oxygen tensions below one atmosphere, a short discussion of HBO toxicity is presented in an effort to elucidate possible mechanisms of oxygen toxicity.

1. Clinical Manifestations

a) Convulsions

Convulsions in laboratory animals and man are the most striking effects of HBO. The work of Bert and Lorrain Smith with small laboratory animals, previously mentioned, has been extended by similar observations of humans in HBO. Experiments on humans have demonstrated a generally greater tolerance to the adverse effects of increased oxygen tension in our species than in most laboratory animals (2).

The first record of convulsions in man was made by Thompson in 1935 (9). He related the experiences of two Royal Naval divers in 100 percent $O_2$ at four atmospheres. The first diver manifested violent twitching of the face after sixteen minutes which disappeared upon breathing air at four atmospheres. The second diver developed tremors of the lips after thirteen minutes and experienced clonic convulsions and unconsciousness after air at four atmospheres was substituted for oxygen. In 1941, U.S. Naval instructions warned (10), "The first signs of oxygen toxicity are flushing of the face, nausea, dizziness, and muscle twitching. A feeling of being irritable and a sense of excitement may follow. As the pressure is increased nausea, vertigo and finally unconsciousness and convulsions ensue."

The rapidity of onset of convulsions is directly related to the $pO_2$ and is accelerated by exercise or the addition of low concentrations of carbon dioxide to the inspired gas (10). The convulsions closely resemble grand mal seizures, with identical EEG patterns, auras, incontinence with post-ictal confusion, amnesia and somnolence (10).

b) Other Manifestations

Other manifestations of HBO toxicity include permanent paralysis and specific CNS lesions in some laboratory animals (5), cytoid body formation in rabbit retinas (11), hemolytic anemia (12), an increased incidence of congenital malformations in rabbit fetuses (13), and pulmonary congestion and edema, cardiac hypertrophy and adrenal hypertrophy in rats (14).

2. Mechanisms of Toxicity

Mechanisms which have been suggested as the basis of HBO toxicity include $CO_2$ accumulation in tissues, altered vascular tone, inhibition of respiration, oxidation of enzyme sulfhydryl groups, inhibition of reduced pyridine nucleotide synthesis, peroxide formation, red cell hemolysis, enhanced tissue radiosensitivity and free radical formation.

a) Increased Tissue $pCO_2$

By raising alveolar oxygen tensions above three atmospheres it is possible to physically dissolve sufficient oxygen (2.3 volumes percent $O_2$ per atmosphere $pO_2$ at 38°C) (15) in the arterial blood so that tissue needs are supplied without utilizing any hemoglobin-bound oxygen. This would preclude the production of deoxyhemoglobin which is responsible for buffering or carrying most of the $CO_2$ produced in the tissues (16). Under such circumstances a rise in tissue $pCO_2$ would be predicted.

Levels of $pCO_2$ of 71-388 mm. Hg. have been reported in sub-

cutaneous gas depots of rats exposed to three atm. $pO_2$ (17). Lambertsen provided evidence (18-20) that these high $pCO_2$ values were artifactual due to faulty sampling techniques (20) and that no appreciable rise in $pCO_2$ occurs until after the onset of convulsions; this rise appears to be primarily due to pulmonary damage and respiratory failure (20).

Working with normal men at one atmosphere, Lambertsen found that 100 percent $O_2$ produced a 15 percent reduction in cerebral blood flow, a one mm. Hg. rise in internal jugular venous $pCO_2$ and no significant change in $CO_2$ production or oxygen consumption (18). In contrast, 100 percent $O_2$ at 3.5 atm. (a $pO_2$ at which convulsions may occur in humans in 15-30 minutes (21, 22)) produces in normal men a 25 percent decrease in cerebral blood flow, a 55 percent increase of cerebral vascular resistance, a 3 mm. Hg. rise in internal jugular $pCO_2$ and an internal jugular venous $pO_2$ of 75 mm. Hg. (normally 38 mm. Hg. $pO_2$ at 1 atm. air) (18). This last figure indicates that some deoxyhemoglobin was present in the internal jugular venous blood of these subjects, whose hemoglobin would be only about 90 percent saturated with oxygen at this $pO_2$. As noted earlier, at 3 atm. $pO_2$, there is enough oxygen in solution to supply the oxygen demands of the tissue provided that regional blood flow and tissue oxygen consumption are unchanged from control levels. In the experiments just mentioned the decreased cerebral blood flow allowed greater oxygen delivery per

volume of blood, enough to produce some venous deoxyhemoglobin.

The (a-v) $pCO_2$ of cerebral blood increased by 8 mm. Hg. in Lambertsen's experiments. Of this increase, 3 mm. Hg. was an increase in internal jugular venous $pCO_2$ and 5 mm. Hg. a decrease in arterial $pCO_2$ due to increased pulmonary ventilation (18). Alveolar $pCO_2$ decreased an average 6.7 mm. Hg. and respiratory minute volume increased 26 percent, due entirely to increased tidal volume (19).

These results indicate that large increases in brain tissue $pCO_2$ do not occur in men exposed to 3.5 atms. $pO_2$. The rise of 3 mm. Hg. $pCO_2$ in internal jugular venous blood is approximately comparable to breathing 5 percent $CO_2$ at one atmosphere (22) which will not by itself produce convulsions (23). The small accumulation of $CO_2$ in brain tissue (reflected by the rise in internal jugular venous $pCO_2$) was interpreted as producing the increased pulmonary ventilation which lowered the arterial $pCO_2$ and this, in turn, produced cerebral arterial vasoconstriction. This mechanism could provide some protection against possible direct toxic effects of elevated $pO_2$ by decreasing arterial blood flow.

That elevated $CO_2$ tensions contribute directly or indirectly to oxygen toxicity is supported by the observation that convulsions begin sooner in animals exposed to HBO when low concentrations of $CO_2$ are added (10, 22, 24, 25), and by the protection against the seizures and pulmonary damage of HBO provided by either hyperventilation (24) or

the intravenous administration of a $CO_2$ buffer like "Tris (tris (hydroxymethyl)aminomethane) (26).

b) Altered Vascular Tone

The decrease in cerebral blood flow observed with an arterial $pO_2$ of 3.5 atmospheres (which is accompanied by a 5 mm. Hg. decrease of arterial $pCO_2$) is approximately equal to the decrease in cerebral blood flow produced by a 5 mm. Hg. decrease of arterial $pCO_2$ alone (19, 27). Therefore, oxygen is probably not important directly in producing cerebral arterial vasoconstriction. Lambertsen demonstrated that no significant decrease in cerebral blood flow occurred with increased inspired and arterial $pO_2$ when alveolar and hence arterial $pCO_2$ were held constant (28). He postulated that $CO_2$ added to inspired HBO increases the rate of onset of convulsions due to a resultant cerebral vasodilation rather than a direct effect of the $CO_2$ (23). That Stadie and Dickens could demonstrate no increased susceptibility of brain tissue slices to toxic effects of OHP upon addition of $CO_2$ (29, 30) fortifies this postulate.

According to Guyton, "Most tissues seem to regulate their blood flow by their need for nutrients rather than by other demands" with the notable exceptions of kidney and brain (31). This concept is demonstrated by vasoconstriction which occurs when the $pO_2$ of Tyrode's solution perfusing a hind limb of the dog is increased (31). Although the retinal vasculature constricts when arterial $pO_2$ is increased, it

appears to be most reactive to changes in arterial $pCO_2$ and dilates when arterial $pCO_2$ is increased (32).

c) Inhibition of Tissue Respiration

Numerous studies have demonstrated toxic effects of 1-3 atm. $pO_2$ on cellular metabolism and respiration (33). Isolated tissues *in vitro* show variations in sensitivity to irreversible respiratory rate depression. The order of sensitivity (high to low) is cerebral cortex, spinal cord, liver, testes, kidney, lung and muscles (34). However, Stadie, Riggs and Haugaard (29) measured normal rates of oxygen consumption by brain tissue taken from rats which had been killed by exposure to 7 atm. $pO_2$; and mice exposed to 8 atm. of oxygen showed no change in rate of respiration, even after the occurrence of convulsions, until only a few minutes before death (35). Thus it appears that *in vivo* inhibition of tissue respiration, in contrast to *in vitro* studies, occurs only very slowly.

d) Enzymatic Inhibition

It has been demonstrated that numerous enzymes are inhibited *in vitro* by HBO and that most of these susceptible enzymes contain sulfhydryl groups (30). Oxidation of sulfhydryl groups to disulfides has been identified in pyruvate oxidase, $\alpha$-ketoglutarate oxidase, NADH-dehydrogenase and LDH (36). Incubation of these inhibited enzymes with cysteine or reduced glutathione restores their activity. This strongly implicates oxidation of adjacent sulfhydryl(-SH) groups to

disulfide (S-S) bonds as the mechanism of inhibition by oxygen.

In addition, some flavo proteins (which contain no sulfhydryl groups in their prosthetic groups (37a)) like xanthine oxidase and cytochrome C reductase are extremely sensitive to oxygen inhibition (33). Nonprotein sulfhydryl groups such as those of cysteine, co-enzyme A and glutathione are rapidly oxidized by oxygen (38).

The presence of heavy metals like copper, iron and manganese is necessary for sulfhydryl oxidation and Haugaard demonstrated protective action of the chelating agent EDTA on cell-free heart homogenates (33).

The significance of enzyme sulfhydryl oxidation in acute oxygen toxicity is uncertain. It has been observed that an enzyme system in the intact cell may be more resistant to oxygen than it is in a cell-free system (21). Also, inactivation of sulfhydryl enzymes occurs to an insignificant degree in the time required for the onset of convulsions (36); and, as mentioned above, inhibition of respiration is not evident in rat brain slices, taken at the time of onset of HBO-induced convulsions.

e) Decrease of Reduced Pyridine Nucleotides

A metabolic change that occurs in vivo rapidly enough to be temporally consistent with its involvement in the etiology of convulsions is the prompt decrease in the tissue concentrations of reduced pyridine nucleotides (NADH or DPNH, NADPH or TPNH) which occurs in rat liver or brain exposed to 2.7 atmospheres $pO_2$ (36, 40). Chance, using

fluorometric measurements of reduced pyridine nucleotide concentrations (41) in intact tissues as well as homogenates, has examined the possible pathways by which this decrease could occur (36). As illustrated in figure 1 (36), NADH may be considered as existing in two pools. Pool #1 is maintained by the action of specific dehydrogenases, probably in combination with NAD (37b), upon intermediates of the citric acid cycle, e.g. $\alpha$-ketoglutarate, which yield reducing equivalents to NAD. The NADH molecules in pool #1 transfer reducing equivalents to the cytochrome system through a flavoprotein dehydrogenase. Succinate is an exception in that it requires energy in the form of high energy intermediates to transfer its reducing equivalents to NAD. Because these endogenous high energy intermediates may be generated by the respiratory chain this pathway is called "reversed electron transfer" in contrast to the "forward electron pathway" which transfers reducing equivalents to ultimately react with oxygen and in the process yields high energy intermediates. The pathway of reversed electron transfer carries reducing equivalents which form NADH pool #2. This pool supplies reducing equivalents for the formation of NADPH and for various metabolic reductions (36, 37b).

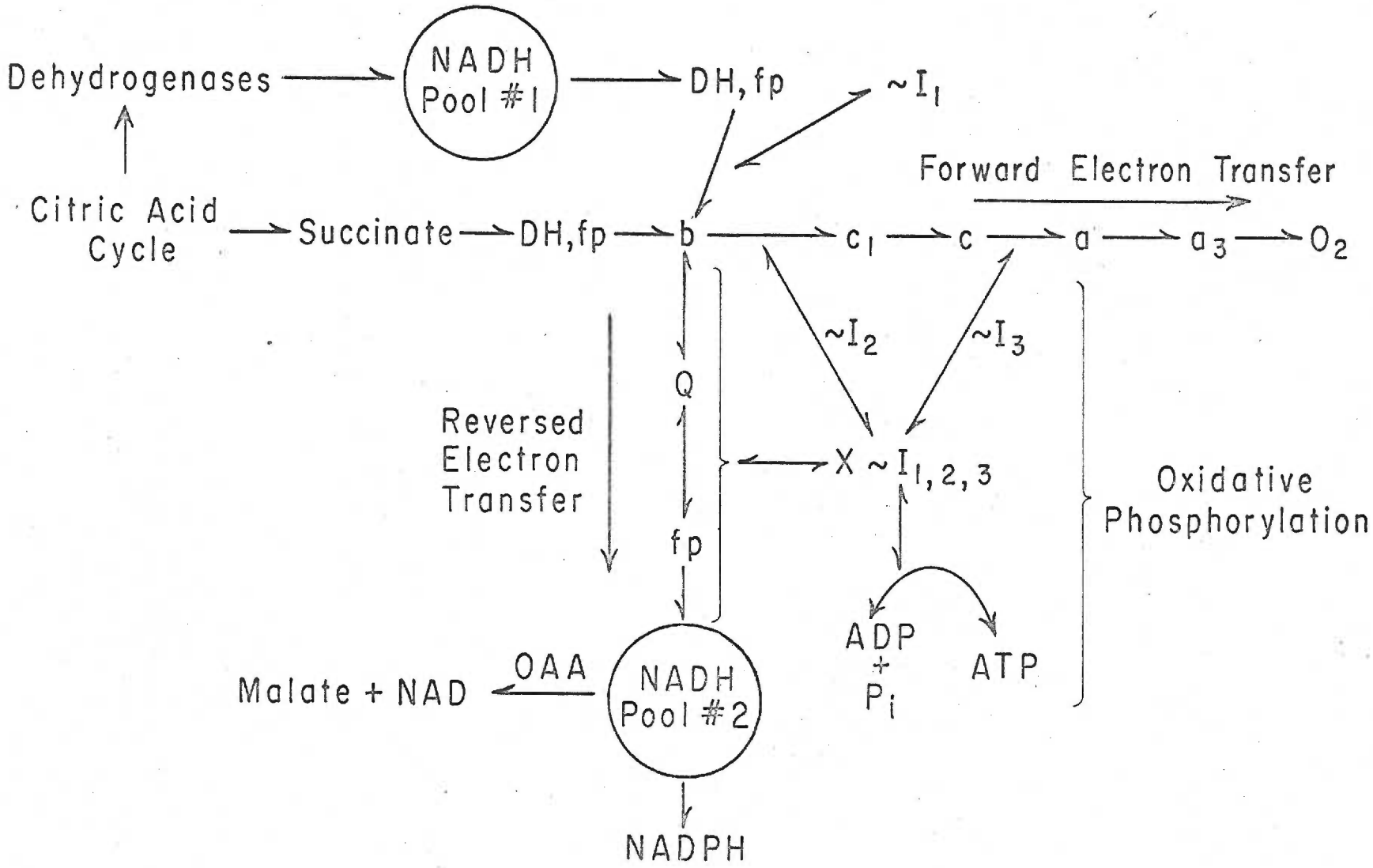
The observed decrease in reduced pyridine nucleotides probably reflects changes in the NADH content of pool #1 or pool #2. This may be considered a result of either activation of reduced pyridine nucleotide oxidation or inhibition of pyridine nucleotide reduction (36.) The former

possibility has been discounted by Chance because (1) the terminal oxidase is the only cytochrome component reactive with oxygen and it is fully saturated at about 0.01 atmospheres $pO_2$, (2) significant direct oxidation of reduced pyridine nucleotides by oxygen was not observed to occur, and (3) formation of free radical forms of the respiratory carriers were not detected.

Inhibition of pyridine nucleotide reduction could conceivably result from oxidation of the sulfhydryl groups of key dehydrogenases to disulfides as has been identified elsewhere (30). However, Chance demonstrated only a very slow and insignificant in vitro dehydrogenase inactivation at 12 atmospheres of oxygen. The possibility of inhibition of electron transport in the respiratory chain was likewise discounted. As indicated in figure 1, the remaining theoretical possibility is inhibition of electron transfer in the reversed direction. Chance believed that the most likely explanation of decreased reduced pyridine nucleotide levels in the presence of high oxygen tensions was inhibition of the energy transfer to the reversed electron pathway which feeds NADH pool #2. These changes do not inhibit the ability of the mitochondrion to carry out oxidative phorphorylation, but do inhibit its ability to transfer reducing equivalents to various acceptor systems (36). This mechanism may explain the observed rise of ATP/ADP levels in liver exposed to high oxygen tensions and suggests that a considerable drain upon in vivo ATP levels may normally occur as a result of the

Figure 1

Pathways of forward and reversed electron transfer. From: B. Chance et al., Control of the oxidation-reduction state of reduced pyridine nucleotides in vivo and in vitro by hyperbaric oxygen. In: Brown, I. W. and Cox, B. G. Proc. of Third Internat. Conf. on Hyperbaric Medicine. Publ. 1404 Nat. Acad. Sci. Nat. Research Council, Wash., D. C., 1966, p. 35.


Dehydrogenases
NADH Pool #1
DH, fp
~I1
Forward Electron Transfer
Citric Acid Cycle
Succinate
DH, fp
b
c1
c
a
a3
O2
~I2
~I3
Q
Reversed Electron Transfer
X ~I1, 2, 3
Oxidative Phosphorylation
fp
ADP + Pi
ATP
OAA
Malate + NAD
NADH Pool #2
NADPH

reversed electron transfer pathway (36). Chance noted that inhibition of an energy-linked reduction would be a unique effect of oxygen: No other known reagent acts on the energy-linked reduction pathway without appreciably inhibiting electron transport and energy transfer in the respiratory chain (40).

f) Red Cell Hemolysis

Red cell hemolysis with HBO exposure has been observed in numerous animals including mice exposed to approximately four atmospheres $pO_2$ for 90 minutes (12). Similar hemolysis can be demonstrated in vitro (12). There appear to be at least three mechanisms of protection against high $pO_2$ in the red cell: sulfhydryl groups in the red cell membrane, reduced glutathione and $\alpha$-tocopherol (vitamin E) within the red cell (43). A reduction in the active concentration of any of these three components predisposes the red cell to lysis when exposed in vitro to systems designed to produce unsaturated lipid peroxidation(12, 43), thus suggesting that HBO hemylosis may involve the lipoprotein membrane of the red cell.

The only known biochemical effect of $\alpha$-tocopherol is inhibition of lipid peroxidation (12). Unsaturated fatty acids readily oxidize in vitro in the presence of oxygen and ferrous ions. This effect is inhibited by the presence of $\alpha$-tocopherol (12). Studies by Kahn and Mengel (12) demonstrated a much greater hemolysis and reticulocyte response in tocopherol-deficient mice following exposure to HBO than

was found in normal mice. Also, tocopherol-deficient mice had demonstrable increases in lipid peroxides, compared to normal controls. These authors suggested that one explanation of HBO hemolysis is the known inhibition by lipid peroxides of acetylcholinesterase. It is known that the osmotic fragility of red cells is increased when acetylcholinesterase activity is inhibited (12).

g) Radiosensitivity

The presence of oxygen usually increases the radiosensitivity of the cell replication process (44). Small increments in oxygen tension (5-10 mm. Hg.) increase radiosensitivity to near maximum levels, without much greater increases in sensitivity at hundreds of millimeters mercury $pO_2$ (44). This fact has led to the use of HBO in tumor irradiation on the premise that normal cells should be little affected by the higher $pO_2$ whereas tumor cells which are believed to be anoxic under ambient conditions should show a large increase in radiation sensitivity under HBO (44). It is estimated that " The presence of molecular oxygen (at its normal pressure in air at sea level) increases by a factor of two to three most of the effects of X- or $\gamma$-irradiation on many living and non-living systems (45)."

h) Free Radicals of Oxygen

Numerous similarities have been noted between the effects of HBO and the effects of radiation (46). Furthermore, some agents, like glutathione, which are known to exert protective effects against ionizing

radiation, will also protect cells against HBO under some circumstances (47).

These striking similarities have led Gerschman to postulate similar mechanisms of toxicity for HBO and radiation. She postulates that both conditions foster formation of free radicals of oxygen. In its usual state, oxygen is ideal as a biological energy source because of its high oxidation potential, abundance, availability and its sluggishness as an oxidizing agent which allows time for storage of energy (8). This sluggishness is possibly due to the activation of oxygen during its reduction into several intermediate free radical states (48). Gerschman believes that rapid formation of free radicals of oxygen (alone or combined with other groups) by HBO not only promotes oxidation directly but possibly results in chain reactions of further oxygen activation.

Although free radicals are well known to exist in biological materials, the actual presence and role of free radicals of oxygen is still poorly defined.

Semi-logarithmic plots of survival times vs. $pO_2$ in mice (8), and of time of onset of toxic symptoms vs. $pO_2$ in men (7) both form hyperbolic curves which approach normal life expectancy with exposure to 20 percent oxygen at one atmosphere. Gerschman speculates that aging processes and life expectancy may reflect the relative degree of "oxygen toxicity" which we experience in our atmosphere (8).

### i) Hormonal Influences

Certain hormones have profound influences upon HBO toxicity. Cortisone, adrenalin, sympathomimetic agents and thyroxin all augment the lung congestion and cardiac hypertrophy characteristic of HBO toxicity in rats (2). On the other hand, either hypophysectomy or adrenalectomy or thyroidectomy or the administration of sympathetic blocking agents will significantly reduce the pulmonary and cardiac manifestations of HBO toxicity (2). Bean believes that immaturity of the adrenals in young animals may explain the greater tolerance to HBO which young animals frequently demonstrate in comparison to old animals (14).

## C. Effects of Elevated Oxygen Tensions of One Atmosphere and Below

The most salient effects of elevated oxygen tensions of one atmosphere or less are pulmonary damage and, in immature animals, retrolental fibroplasia. These two effects have received the most investigative attention. Less well studied effects include metabolic and growth alterations and effects on the cellular elements of blood.

### 1. Pulmonary Effects

Lorrain Smith (4) first described dyspnea and subsequent death in rats exposed to 70-80 percent oxygen at one atmosphere for four to eight days and observed pulmonary congestion, consolidation and edema at autopsy in these animals. He likened the effects of 70-80 percent

oxygen at one atmosphere for four to eight days to those of three and one-half atmospheres of 100 percent oxygen for five hours. He could detect no effect of 40 percent oxygen on rats after eight days. Since these classic experiments many details have been added, but Smith's observations still provide the core of our understanding about effects of increased oxygen tensions below one atmosphere on the lungs.

Similar changes have been observed in a wide variety of animals exposed to oxygen concentrations greater than 60 percent for prolonged periods (24). The pulmonary changes observed include capillary congestion; alveolar proteinaceous exudates; intraalveolar hemorrhage and fibrinous exudates with prominent hyaline membranes lining alveolar walls, ducts and respiratory bronchioles (49); pulmonary alveolar cell hyperplasia and pulmonary arteriosclerosis (24); edema of alveolar septae and atelectasis (50); sparse chronic inflammatory exudates, fibroblastic proliferation, early fibrosis (49) and decreased tracheal mucous flow (51).

As is true with HBO, oxygen at less than one atmosphere pressure demonstrates various degrees and kinds of toxicity within and between species, at different oxygen tensions and with different lengths of exposure. For example, rats exposed to 98 percent oxygen (an alveolar $pO_2$ of approximately 650 mm. Hg.) for 48 hours showed interstitial edema and destruction of pulmonary capillary endothelium, while rats exposed to the same oxygen percentage for 72 hours showed

exudates of leukocytes, red blood cells and fibrin in the alveolar walls (24). Dogs exposed five to fourteen days to environmental oxygen pressures of 300-350 mm. Hg. demonstrated only drowsiness and at autopsy, alveolar wall thickening; whereas dogs exposed tc oxygen tensions above 350-400 mm. Hg. suffered fatal pulmonary injury in two to six days in nearly every case (53).

Man is generally considered to have a greater resistance to the pulmonary manifestations of oxygen toxicity than most laboratory animals (24). Of 90 healthy adult volunteers exposed to 97-99 percent oxygen for 24 hours, 82 percent had substernal distress; nose and throat irritation was common, as was a decrease in vital capacity. Symptoms frequently occurred after only six hours of such exposure. No effects were noted with 50 percent oxygen (54).

Pratt has found pulmonary alveolar congestion, thickening of alveolar septa and pulmonary capillary proliferation with projections of capillary tufts into the alveolar spaces at autopsy of patients exposed for two to seven days prior to death to oxygen by nasal catheter (55).

In contrast, 100 percent oxygen at a total ambient pressure of 250 mm. Hg. has been breathed by humans for 30 days without detectable pulmonary changes (56). One hundred percent oxygen at an ambient pressure (187 mm. Hg.) such that the alveolar $pO_2$ is not greater than normal, produces no evidence of toxicity (25), but pre-

sumably rapid absorption of gaseous water, carbon dioxide and oxygen could cause atelectasis in bronchioles occluded even by normal secretions when pure oxygen is breathed even at such low pressures (25).

Although it has been frequently stated that 60 percent oxygen (an alveolar $pO_2$ of approximately 350 mm. Hg. ) is the threshold for danger (24), symptoms of substernal distress have been reported in one out of five individuals exposed to an environmental $pO_2$ of 190 mm. Hg. for fourteen days, and in one out of eight individuals exposed to an environmental $pO_2$ of 174 mm. Hg. for 17 days (57).

Damage to the alveolar capillary wall may represent the first injury produced by oxygen inhalation, leading to alveolar edema and hemorrhage and the other changes characteristic of pulmonary oxygen toxicity (52, 58). A demonstrated decrease in pulmonary surfactant (50) probably contributes to the patchy atelectesis which is frequently observed. A decreased activity of proteolytic enzymes from the alveolar cells may underlie the abnormal accumulations of alveolar protein which are observed (59).

That bacterial infection or overgrowth is not an essential factor in the pathogenesis of oxygen toxicity to the lungs has been established by the production of similar changes in germ-free rats exposed to 100 percent oxygen (60).

Pulmonary toxicity resulting from 100 percent oxygen at one atmosphere, as evidenced by pulmonary congestion and survival times,

is increased by the administration of thyroid (61) or cortical or medullary adrenal (62) hormones. Conversely, either thyroidectomy or hypophysectomy permits increased survival of rats exposed to elevated oxygen tensions compared to intact normal rats with similar exposure (54).

Intermissions of normal air breathing between exposures to elevated oxygen tensions prolong survival and ameliorate symptoms resulting from such exposure (54, 63).

2. Retrolental Fibroplasia

Retrolental fibroplasia (RLF) is another manifestation of the toxicity of oxygen at tensions below one atmosphere. In such animals as premature human infants or newborn mice, rats and kittens, whose retinal vasculature has not completed its growth, prolonged exposure to elevated oxygen tensions followed by return to room air frequently results in disordered retinal vascular proliferation and neovascularization of the vitreous. These changes lead to retinal detachment, retinal and vitreous hemorrhages and consequent blindness.

RLF occurs in premature infants weighing less than 1500 grams if they are exposed during the first ten days of life to oxygen concentrations of 50 percent or more at normal atmospheric pressure (64). Between 1943 and 1953 nearly 7000 premature infants were unwittingly blinded because of therapeutic exposure to elevated oxygen tensions, with total detachment of the retina and formation of a dense fibrous

membrane behind the lens. Many other premature infants so exposed developed incomplete RLF with varying degrees of visual damage (64). Unexplained, however, is the fact that from 0.25 to 0.50 percent of RLF victims have received no significant oxygen therapy (65).

Ashton described the effects of elevated $pO_2$ upon the retinal vessels of newborn kittens following exposure to 60-80 percent oxygen at one atmosphere for four to six days (66). In kittens, the retinal vessels do not reach the retinal periphery until about three weeks after full term birth (66); in contrast, the retinal vasculature of the full term human infant is complete at birth (64) or shortly thereafter (65). The developing retinal vessels of the kittens were obliterated, and quoting Ashton, "Some of the collapsed vessels contain trapped coagulated blood. After transfer to air for three days, such vessels as remain patent appear to reopen giving rise to a grossly abnormal cobweb of capillaries.... The reestablished blood supply appears to be totally inadequate for the requirements of the retina when the animal is breathing air, and vessel growth into the ischemic retina recommences from the disc region (66)." Ashton noticed that "... the extent of vascular obliteration by oxygen is directly proportional to the degree of immaturity of the retinal vascularization, to the duration of exposure to oxygen, and to the degree of oxygen concentration (66)." The fully mature retinas of older kittens did not show these structural abnormalities following similar oxygen exposure. Ashton concluded that,

"The process of vasoobliteration by oxygen represents an exaggeration of capillary retraction, which is a recognized component of normal capillary growth, wherein the endothelial cytoplasm and nuclei of redundant capillaries retract into the parent capillary.... (67)."

Patz described similar retinal changes without systemic effects in newborn rats who breathed 80 percent oxygen for three weeks (68).

Patz noted no effects in rat mothers or their fetuses when pregnant rats were exposed to 90 percent oxygen at one atmosphere for 48 hours or 100 percent oxygen at two atmospheres for six hours at various gestational states (68). However, Fujikura exposed pregnant rabbits at 26 and 27 days gestation alternately to 97-100 percent oxygen at one atmosphere and 97-100 percent oxygen at 1.2 atmospheres at ten minute intervals over 15 hours. He observed a high incidence of stillbirths, microophthalmia, retinal detachment and intraocular hemorrhage as well as marked congestion of the lungs and other organs in the offspring delivered normally two days later. The exposed mothers were not noticeably affected; pregnant rabbits cycled in room air produced offspring who were relatively free of pathologic changes (69).

Strikingly similar to the vascular responses of the immature retina (an embryonic derrivative of the forebrain (70)) are the vascular changes noted in the cerebral cortices of newborn mice exposed to 90-100 percent oxygen for 5-30 days (71). Continuous exposure to such

an environment produces mice of significantly smaller size and with cerebral cortical vascular underdevelopment, even when compared to control mice of similar weight. From measurements of the total length of vessel fragments in a given area of cerebral cortex, Gyllensten found that following a five day exposure to 100 percent oxygen, transfer to room air produced such an accelerated proliferation of vessels that the vascular development of the experimental animals soon exceeded that of the control animals by a significant five to ten percent. Interestingly, at fifteen days, the vessels of the visual cortex (only) decreased compared to control animals - an effect considered by the authors as secondary to the retinal damage produced by the elevated $pO_2$ (71).

### 3. Respiratory and Circulatory Effects

Upon initial exposure of resting human volunteers to 100 percent oxygen at one atmosphere, there is a transient decrease in ventilation. At this stage there is a decreased ventilatory responsiveness to inspired carbon dioxide and the ventilatory response to carbon dioxide progressively decreases with further elevations of $pO_2$ (25). Prolonged oxygen breathing at one or more atmospheres by normal men at rest leads to increases of respiratory minute volume. This may be the result of a rise in carbon dioxide tensions and hydrogen ion concentrations in brain tissues due to the decreased transport of carbon dioxide. According to this concept brain tissue acidosis and arterial alkalosis

exist concurrently (25). The studies of Lambertsen et al. (18) demonstrated that inhalation of 100 percent oxygen at one atmosphere produced a 15 percent decrease in cerebral blood flow, a one mm. Hg. rise in internal jugular venous $pCO_2$ and no significant changes in oxygen consumption or carbon dioxide production. From Bean's extensive review (24), it appears that most of the studies on man and various other animals between 1900 and 1945 demonstrated no change in oxygen consumption with roughly similar elevations of oxygen tension. Comroe et al. (54) studied 31 normal humans breathing 100 percent oxygen at one atmosphere for 24 hours and detected no consistent change in blood pressure or pulse rate with exposure to elevated oxygen tensions (54).

4. Hematologic Effects

The effects of elevated oxygen tensions upon cellular elements of the blood have only recently been clarified (24, 72). Acute exposure to oxygen tensions elevated to less than one atmosphere has little or no detectable effect upon the hematocrit, red blood cell count or indices (24, 54), but prolonged exposure of from several days to several weeks produces subtle changes. Linman and Pierre (72) exposed mice to four atmospheres of compressed air ($pO_2$ approximately equal to 650 mm. Hg.) and demonstrated a decline in RBC $^{59}Fe$ incorporation within 24 hours, reaching 20 percent of the previous normobaric value at 72 hours. Reticulocytopenia, with reticulocytes averaging 50 percent of

normobaric values was demonstrable in 72-96 hours. However, no change in hematocrit or other red blood cell parameters was detected for periods up to eight days. The administration of erythropoietin to the hyperbaric mice at five and again at six days restored $^{59}Fe$ uptake into red blood cells to about 60 percent of the normobaric value, suggesting suppression of hormonal erythropoietic stimulation by the elevated oxygen tension. No hemolysis was detectable although hemolysis has been found in men breathing 100 percent oxygen at 7.4 psi. (approximately 380 mm. Hg.) after 48 hours (56). Exposure of rats to 600 mm. Hg. oxygen for 28 days has produced significant decreases in hematocrit (a decrease of 20 percent), red blood cell count (down 13 percent), mean corpuscular volume (down 7 percent) and mean corpuscular hemoglobin (down 11 percent) (73).

Effects on other organs have been noted. Oxygen at 0.7 atmosphere produced injury to testicular germinal epithelium after three to four weeks (8). Gerschman speculated that the retinopathy observed in young vitamin E deficient rats who were exposed to room air may be an example of oxygen toxicity in animals lacking normal antioxidant defense mechanisms (8). Stimulant (74) as well as inhibitory (8) effects have inconsistently been noted during embryonic development in various animals. The effects of elevated oxygen tensions upon the growth of chick embryos will be discussed in a later section.

5. Mechanisms of Toxicity of Oxygen at Pressures Less Than One Atmosphere

Biochemical mechanisms of the pathogenesis of oxygen toxicity are essentially unknown. Elevated oxygen tensions less than one atmosphere mimic many of the biochemical effects of HBO. Exposure to 100 percent oxygen at one atmosphere reduces CoA content (75) of rat brain in vivo and the rates of carbohydrate metabolism of heart and brain homogenates: There is a decreased utilization of glucose and $\alpha$-ketoglutarate and a decreased formation of high energy phosphates (76). These effects are enhanced by the addition of $Cu^{++}$ or $Fe^{++}$ions and diminished by the addition of EDTA. This is consistent with the hypothesis that disulfide formation is involved in the inhibition of glycolytic enzymes containing sulfhydryl groups since the presence of $Cu^{++}$ions is required for disulfide bond formation (76). One hundred percent oxygen at one atmosphere produces slow inhibition of some enzymes (many containing sulfhydryl groups) and slow depression of respiration of brain homogenates and brain slices (30, 77). Studies of homogenates must be viewed cautiously because it has been observed that an enzyme system when present in the intact cell may be more resistant to oxygen than when exposed in cell-free systems (21). This may be related to the demonstrated (77) protection of enzymes by their coenzymes or substrates (33).

An entirely different interpretation of the toxic effects observed with elevated concentrations of oxygen, is that these effects result from a lowered nitrogen tension rather than from an increased oxygen tension.

Allen (78) studied the vascular development of chick embryos exposed to different gas mixtures. If the $pO_2$ was kept around 150 mm. Hg. normal vascular growth occurred as long as the $pN_2$ exceeded 80 mm. Hg.; chicks exposed to a $pN_2$ below 80 showed marked inhibition of vascular growth even when the $pO_2$ was kept at 150 mm. Hg., an effect closely resembling that produced by 100 percent oxygen at one atmosphere.

An experiment comparing incubation in 21 percent oxygen and 79 percent helium, with room air controls, demonstrated a 50 percent decrease in the percentage of chicks hatching and an 8 percent decrease in hatching weight in the helium-oxygen eggs (79). The chicks incubated in the helium-oxygen mixture had the same total nitrogen content at 16 1/2 days as the controls (79).

Volskii (80) described a marked increase of early embryonic death in experiments where nitrogen was replaced by helium, argon or xenon. Whole eggs demonstrated progressive elevations of total nitrogen content during incubation with a four percent elevation at the time of hatching, and parallel rises in uptake of $^{15}N$ over the embryonic growth period. These data suggest that a low partial pressure of nitrogen may produce an effect resembling that associated with elevated oxygen tensions. Because elevated pressures of room air (an atmosphere with above normal $pN_2$ as well as $pO_2$) will produce oxygen toxicity (3), it appears unlikely that a low $pN_2$ will explain all

oxygen toxicity; rather, two kinds of toxicity may be responsible for "oxygen toxicity", i.e. low $pN_2$ and/or high $pO_2$.

Disparate results were obtained by Wright et al. (81); using a helium-oxygen environment they found no significant difference in dry weights at any one age, and both experimental and control eggs had the same percent of normal embryos. Savin et al.(82) found that replacement of 99 percent of the atmospheric nitrogen with helium did not prevent normal development of chick embryos.

Another interesting study is that of Mac Hattie and Rahn (83) who maintained mice for nearly two months in 100 percent oxygen with nitrogen and carbon dioxide maintained at less than 4 mm. Hg. The animals were placed in a sealed enclosure with food, water and gas supplied and the seal was not broken for 51 days. Several females became pregnant during the experiment and delivered at the appropriate times; their offspring were healthy and appeared to develop normally in the nitrogen-poor environment.

The controversy regarding low $pN_2$ as one mechanism of toxicity is not settled at present. The toxicity of reduced nitrogen tension is an interesting, though iconoclastic, hypothesis. It challenges the widely held belief which appears to be based largely upon one unpublished observation by Lavoisier (84) that nitrogen is neither chemically consumed nor produced in the animal body. Recent experiments (84) have demonstrated increases in the concentrations of

gaseous nitrogen in the environments of germ-free rats. These findings are taken as evidence of elemental nitrogen production and strengthen the challenge to our general concept of gaseous nitrogen as a biologically inert gas in animal metabolism.

D. Capillaries

1. Morphology

Capillaries are cellular tubes which convey blood through tissues from arterioles to venules and provide a large surface area for exchange between the extra- and intravascular spaces. Various specializations in capillary morphology occur in certain areas like hepatic sinusoids and renal glomeruli (85). The following description is of a typical muscle capillary which can serve as a prototype for a discussion of such variations.

The questions concerning the presence or absence of intercellular cement between endothelial cells, of whether basement membranes surround the endothelial components, and about the porosity of interendothelial spaces which concerned earlier light microscopists (85) have been considerably resolved with the aid of the electron microscope. Capillaries are now considered to have three layers (86), endothelial, middle and adventitial. The inner layer is made of flattened endothelial cells varying in thickness from four or five micra in the thicker nuclear regions, to 0.5 micra in the more attenuated portions of the cells (85). The inner cell membranes are in direct contact

with blood and exhibit an irregular surface with frequent short microvilli and blunt pseudopodial projections. Thin projections, called marginal folds, are visible near the cell margins and may be involved in pinocytosis (85). The cytoplasm of endothelial cells contains vesicles which in the attenuated peripheral parts of the cells may occupy up to 18 percent of the cytoplasmic volume (86) and seem to be important in transcapillary exchange (87). Contrary to expectations based upon light microscopy, the thin peripheral parts of contiguous endothelial cells are not joined edge to edge by a conspicuous intervening layer of intercellular cement. Instead, intercellular margins are usually very tightly apposed with imbrications and interdigitations of varying complexity. The cell margins are seldom more than ten mμ. apart; the intercellular space may be occupied by a homogeneous material of moderate density (88, 89). Adjacent cells are joined along the luminal one-third of their intercellular margins by narrow belts of membrane fusion, the zonulae occludentes which circumscribe the entire endothelial cell (86, 90). Such observations cast doubt on earlier theories of intercellular pores which were postulated in order to account for the transcapillary exchange of substances (85). Studies of the transcapillary exchange of ferritin molecules ($\approx$ 11 mμ. diameter) revealed no ferritin in intercellular junctions nor within the cytoplasmic matrix; rather, ferritin was restricted to cytoplasmic vesicles and the extravascular spaces (87).

Applied to the outer surface of capillary endothelial cells is a continuous layer of moderately dense material 30-50 mμ. in thickness, the basement membrane or, more correctly, the basement lamina (85). Ferritin appears to pass the basement lamina relatively easily (87), but the lamina probably has some of the qualities of an ultra filter and may have an influence upon capillary permeability (85).

The basement lamina envelops the endothelial cells and splits into inner and outer leaves to include the pericytes (86). These cells have an elaborate system of processes, usually disposed perpendicularly to the long axis of the vessels, which penetrate the inner leaflet of the basement lamina and fuse to the endothelial cell membranes (86). The function of the pericytes has long stirred interest and controversy among physiologists and anatomists (85, 86, 91, 92, 93). Together the pericytes and basement lamina comprise the middle layer of capillary wall (86).

The outer layer, or adventitia, is a discontinuous layer of macrophages, fibroblasts, mast cells, extracellular fibers and amorphous matrix elements, similar to and continuous with the components of the pericapillary spaces (86).

Capillaries appear to be dynamic and labile structures. Newly formed capillaries are capable of segmental involution and rearrangement even with slight stimuli (92). Constant endothelial DNA synthesis occurs in mature mouse capillaries at a rate which has led to an esti-

mate of endothelial turnover time of about three years (94).

Individual capillaries have frequently been observed to open and close completely (93), suggesting the possibility of endothelial or pericyte contractility. Capillary control seems difficult to explain solely on the basis of precapillary arteriolar sphincters (95). However, the present consensus is that in higher vertebrates neither endothelial cells nor pericytes are actively contractile (92, 93, 95), and that capillaries preferentially open and close by the hydrostatic influences of arteriolar constriction (95, 96). Clark observed active capillary contractility in newly formed capillaries of amphibian larvae after two days; the contractility appeared to be independent of pericytes (92). He detected no contractility in capillaries of the rabbit ear (92). Capillaries of the chick area vasculosa may also be contractile (97).

2. Capillary Development

According to Arey, "The earliest vascular primordia in most vertebrate embryos are clusters of cells arising on the yolk sac, between the splanchnic mesoderm and entoderm. These blood islands are at first compact masses, but they soon separate into peripheral cells that become endothelium and into more centrally located cells that produce a short lived line of primitive blood cells (88)." With the appearance of blood islands, this area of the blastoderm is termed the area vasculosa (98a). It is first evident in the chick at about the

four somite stage, or after approximately 26-29 hours of incubation (98a), and in the human embryo toward the end of the third week following fertilization (69). Endothelial cells proliferate within the blood islands and form a plexus of capillaries which progressively extends both over the yolk sac and centrally toward the embryo. However, these vessels are not the source of endothelium for the embryo (88). Mesenchyme within the embryo differentiates locally into endothelium (88, 69, 99) and through a process of sprouting and longitudinal growth localized capillary plexuses form and enlarge. Leading endothelial cells join their own kind of cell (100) and then anastomose with adjacent capillary plexuses to form continuous vascular channels, even in the absence of a circulation (92). These capillary plexuses are the primordia for nearly all the definitive vasculature. Only in a few instances, such as the dorsal segmental branches of the aorta, do definitive vessels first occur as single tubes (88).

Clark observed that newly formed vessels in the tail of the frog tadpole were initially bare endothelial tubes and that eventually cells resembling fibroblasts migrated to the capillary wall and spread out upon it, becoming pericytes (101). Recent studies of mammalian retinal capillary formation (102) suggest that two mesenchymal cells join and form a basement membrane between them with the inner cell becoming the endothelial cell and the outer cell the pericyte. Other studies (96) also suggest that pericytes derive from mesenchymal cells,

fibroblasts or macrophages. Retinal capillaries appear to arise either by budding from the hyaloid vessels (96) or formation of cords from undifferentiated cells of the outer wall of the hyaloid vessels (103). These solid cords of cells are preceded into the inner layers of the retina by undifferentiated spindle cells with characteristic glycogen granules and a high mitotic rate (104). These cells disappear as the cords acquire lumina and are absent in the mature retina (96).

Ashton has recently reported that cultures of young rabbit endothelial cells show cell disintegration with the simultaneous appearance of mesenchymal cells (possibly derived from the endothelial cells) when the culture is from 24 to 48 hours old. After 48 hours, the mesenchymal cells interconnect to form an irregular network; over several days the cells assume a parallel alignment with delicate branching cords which gradually differentiate into well-formed capillary tubes. In addition, some mature endothelial cells proliferate into columns without passing through a preliminary mesenchymal stage (96). Similar endothelial dedifferentiation with subsequent capillary formation was noted by Savin in a study of healing intestinal anastomoses in adult dogs (105).

Other studies of wound repair have shown capillary development similar to that of the developing embryo or immature retina and suggest that capillary plexuses grow by two processes. The highest

mitotic rate is generally observed at the proximal end of a growing capillary plexus (106, 107) where growth occurs principally by endothelial proliferation. At the distal advancing end of a capillary sprout growth is principally by distal migration of endothelial cells. Low grade stimuli or mild injuries induce expansion of pre-existing vascular networks by endothelial proliferation and intercalation of these cells without sprout formation; in contrast, strong stimulation produces growth by sprouts (106, 107). These sprouts grow rapidly for the first four or five days then the growth rate gradually declines (92). The capillaries are accompanied throughout their growth by macrophages and fibroblasts (92).

Newly formed capillaries are very permeable and fragile (92, 96, 106, 107) and the endothelial intercellular junctions are wide and irregular although zonulae occludentes are frequently noticeable (106); the basement laminae are thin and often discontinuous (107). The endothelial cytoplasm shows increased cytoplasmic inclusions, richly granular endoplasmic reticulum and numerous large mitochondria (107) - evidences of high cellular activity. Differentiation into arteries and veins is detectable in the older parts of a newly formed capillary plexus (92). Pericytes retain their primitive potential and will differentiate into smooth muscle cells under the stimulation of increased blood flow (108). Injection of small glass beads into the carotid arteries of kittens results in numerous interarterial shunts in the newly

formed retinal capillaries. As some capillaries experience an increased blood flow they acquire the function of arterioles; intramural pericytes proliferate and form the simple muscular coat of normal terminal arterioles (96).

### 3. Control of Capillary Growth

The growing capillary plexuses do not extend equally into all parts of the embryo and appear to be influenced by the presence (or absence) of some unidentified angiotaxic factor(s) (88). An originally non-vascular tissue like the neural tube attracts a close vascular net, but, the lateral sides of the tube become vascularized while the roof and floor remain non-vascular (88).

The irregularity of the surface upon which cells migrate also influences their rate and direction of movement (100). Generally, vessel formation tends to follow pathways of least resistance, through tissue clefts and spaces (108).

Once capillary plexuses are formed, a process of selection occurs which results in permanence and enlargement of some channels and obliteration of others (92, 96). Several factors governing these events have been postulated (88). One is heredity.

A heart beat and blood flow are usually necessary for the maintenance of smaller vessels (88), but the great veins and arteries can develop and exist for a time in the absence of a beating heart, although they cannot attain and maintain a normal differentiation (109).

Other factors include such physical forces as the blood volume, the rate of blood flow and the blood pressure. Even in areas of new vessel formation capillary retraction occurs if blood flow decreases (92). If the basement lamina has already formed a bridge of this material will persist. The endothelial cytoplasm and nuclei retract into the cells of parent capillaries (96) or an endothelial cell may migrate and spread itself upon the endothelial cell of an existing channel as a pericyte (101).

A set of propositions stated by Thoma in 1893 (110), and known as "Thoma's laws" are (from Arey's translation (88)):

1. Diameter of a vessel depends upon the rate of blood flow.

2. Length of a vessel depends upon the longitudinal tension exerted on the vessel by surrounding tissues and organs.

3. Wall thickness depends upon the blood pressure.

4. New capillary formation depends upon the blood pressure.

The first three propositions are in general agreement with the findings of Clark and Clark (92) and Ashton(96). But, as the Clarks point out (92), blood pressure is not an adequate explanation of new capillary formation because new capillaries will send out sprouts which anastomose to form a plexus in the absence of a circulation, as after the removal of the heart in an embryo (109). Also, as noted by the Clarks, if blood pressure were the determining factor in the formation of new capillaries after their primary differentiation, a greater

number of new sprouts would arise on the arterial side than on the venous, which is contrary to the observations of Clark (109) and Ashton (96).

Besides genetic, mechanical and hemodynamic influences, there appear to be others which are important in regulating capillary growth. Clark observed that promoters of blood vessel growth also stimulated growth of lymphatics, nerves and connective tissue. A localized inflammation appeared to stimulate capillary growth (92) but the mere presence of leukocytes did not seem to stimulate capillary growth (111).

Studies of wound healing have shown that higher than normal levels of circulating cortisone (112) will decrease the rate of wound healing as will ascorbic acid deficiency or protein depletion. Methionine is the only sulfur containing essential amino acid and is important in connective tissue synthesis (113a). In methionine deficient animals, wounds show a marked decrease in capillary budding and fibroblastic activity (113a). Cortisone administration will decrease the rate of expansion of collateral circulation following arterial occlusion (112).

There is evidence which suggests that the new growth of tissue during repair may be due to the absence of a growth inhibitor (113b). If two epidermal sheets are separated by a sheet of connective tissue and cartilage 0.1 mm. thick, removal of a 3 mm. disc of epidermis on one side noticeably stimulates growth in the epidermis on the other

side and the highest growth stimulation is in the area opposite the center of the lesion.

There are numerous observations which suggest that a decreased oxygen tension may stimulate capillary growth. Rats maintained at high altitude evidence an increased capillary density and diameter in the cerebral cortex (114), and guinea pigs native to the Peruvian mountains show increased capillary density in striated muscle (115). Anemia, coronary artery occlusion or marked coronary artery stenosis are each associated with greater collateral circulation in the human myocardium (116). The changes of retrolental fibroplasia (66, 67, 68, 69, 96, 108) in animals with an immature retinal vasculature and the very similar changes observed in the cerebral cortex of a newborn mouse (71) suggest that exposure to elevated oxygen tensions exerts a strong inhibitory influence upon normal capillary growth; upon return to room air, a sudden relative hypoxia is postulated which provides a potent stimulus for capillary and fibroblastic growth overriding normal control mechanisms and resulting in a disorganized, profuse capillary proliferation into the adjacent vitreous, as well as the retina (96). Injections of small glass beads into the retinal arteries of kittens results in a vascular proliferation exactly like that seen after return to room air following exposure to elevated oxygen tensions (108).

Ashton (96) believes that the pathologic responses of retinal capillaries seen in RLF and retinitis proliferans (96), are exagerated

forms of normal capillary behavior. Similarly, the responses of the retinal vasculature to high oxygen tension resemble normal capillary behavior. Kittens in 80 percent oxygen demonstrate retinal vasoobliteration and capillary closure followed by degenerative changes in endothelial cells and subsequent retraction and migration of endothelial cells to surviving capillaries (96) - a process similar to capillary plexus formation and modification which has been described earlier. The development of retinal edema is not important in the process of capillary closure because this will occur in the young rabbit retina while the retinal vessels still lie entirely upon the surface of the retina (96). The vascular effects of high oxygen exposure which characterize retrolental fibroplasia appear to be limited to immature retinal (96) or cerebral cortical (71) capillaries; the abruptness of removal from the elevated oxygen atmosphere is much less important than the total exposure time (117).

Other vascular responses to elevated oxygen tensions are observed after chronic exposure to increased oxygen. The developing gills of one species of salamander (Salmandra maculosum) had shorter, thicker filaments and a decreased blood volume when the animals were exposed to 100 percent oxygen at one atmosphere pressure compared to members of the same species who were exposed to 10 percent oxygen at one atmosphere. The effects noted were primarily due to changes in cell size rather than mitotic rate. Another salamander

(Ambystoma opacum) manifested little change with similar exposures (118). Flemister and Cunningham (119) injected the chorioallantoic vessels of developing chick embryos with radioopaque dye and observed the gross vascularity of this membrane on roentgenograms. Chicks exposed to about three atmospheres of air pressure had a noticeably depressed vascular development. Allen (78) studied chick embryos after four days of incubation in 100 percent oxygen and observed marked inhibition of development of vessels in both embryo and yolk sac.

Two interesting observations which are possibly physiological adaptations to variations of oxygen tension are worthy of note. One is the arrangement of capillaries so that there are much fewer on the arteriolar than venular side as noted by Ashton (96) in retinal vessels. The other is the sustained oxygen consumption of arterioles at oxygen tensions up to 150 mm. Hg., while the oxygen consumption of venules falls markedly as the oxygen tension approaches this level (120).

E. Effects of Variations of Oxygen Tension Upon Chick Embryos

Studies of the percentage of eggs which eventually hatched (hatchability) when incubated at different oxygen concentrations and one atmosphere total pressure have produced a variety of results. Taylor et al. (121) found no significant increase in hatchability when oxygen concentration was raised above 20 percent, a decreased hatch-

ability at oxygen concentrations above 75 percent and a sharp decrease below 18 percent, with extinction near 10 percent. Barrott (122), found a decrease of approximately one percent in hatchability for every one percent increment of oxygen concentration above 21 percent. Cruz and Romanoff (123) found that the highest hatchability occurred at 32 percent oxygen decreasing on both sides of this concentration. In addition they noted that up to the fifth day the growth rate was most rapid in 40 to 70 percent oxygen, although this group had a low hatchability. The one thing that all three studies agree upon is that chick hatchability, and hence mortality, is unfavorably affected by oxygen concentrations above 75 percent.

A study (124) of hematologic values in chick embryos exposed to 15, 21 and 40 percent oxygen concentrations demonstrated no difference between 21 and 40 percent oxygen, but 15 percent oxygen produced an increased red blood cell count compared to the blood of chick embryos incubated in 21 percent oxygen. Flemister and Cunningham (119) incubated chick embryos at three atmospheres of room air and described a 40 percent increase in weight at ten days and a 60 percent decrease in hemoglobin concentration. Allen (78) found a marked inhibition of vascular development during the first four days if chick embryos were incubated either in 100 percent oxygen or at a partial pressure of nitrogen less than 80 mm. Hg.

Biochemical variations which occur in the chick embryo in

response to different incubator oxygen tensions include changes in the proportions of lactic dehydrogenase (LDH) isozymes. There are five known isozymes of LDH, numbered one to five (125). $LDH_1$ predominates in relatively aerobic tissues like heart and brain; $LDH_5$ predominates in tissues which tolerate anoxia, like skeletal muscle. Chick embryos incubated in 15, 20 and 40 percent oxygen at one atmosphere showed no differences in total LDH activity; but those incubated in higher oxygen concentrations had higher $LDH_1$ activities and those incubated at lower oxygen concentrations had higher $LDH_5$ activities when their tissues were compared with those of controls incubated in 20 percent oxygen.

Several molecular forms of hemoglobin have been identified in the chick embryo. Normally hemoglobin I predominates early in the incubation period with hemoglobin II becoming the major component by the time of hatching (126). In ovo oxygen deficiency retards the usual shift from hemoglobin I to hemoglobin II and in vitro oxygen deficiency accelerates heme synthesis and preferentially the synthesis of hemoglobin I(126). Effects of elevated oxygen tensions upon hemoglobin synthesis were not described.

This writer knows of only two studies which bear directly upon the subject of the present investigation.

The first is that of Flemister and Cunningham (119) who injected the chick chorioallantoic vessels with radioopaque dye and compared

the roentgenograms of eggs at various stages of development. When eggs were incubated in room air at 40 pounds of pressure (a $pO_2$ comparable to 57 percent oxygen at one atmosphere) and compared with those incubated at one atmosphere of room air (14.7 lbs.) a decrease in chorioallantoic vascularity (to gross vision) was noted in the high-pressure eggs and at the end of ten days the major allantoic vessels had progressed only to the stage normally attained after eight days.

The other study is that of Remotti (127) who observed the chorioallantoic vasculature of chick embryos incubated in 50-75 percent oxygen at one atmosphere. By the sixth day, it was noted that individual chorioallantoic capillaries were wider in diameter than normal and arranged in wider meshes, changes which together tend to reduce the total area available for gas exchange.

F. The Egg Shell and Membranes

The following description is to orient the reader regarding the formation of the chorioallantoic vascular membrane and the nature of the diffusion barriers between capillary blood and the external atmosphere.

During the fourth day of incubation the allantois, arising as a diverticulum of the hindgut (98b, 128a), emerges on the right side of the ventral surface of the embryo as a progressively enlarging sac occupying the extraembryonic body cavity (98b). The sac is composed of an inner entodermal layer and an outer mesodermal layer. At about

100 hours, the mesodermal side of the allantoic sac meets and begins fusion with the inner, mesodermal layer of the chorion (which has an outer layer of ectoderm) to form a three-layered membrane composed of ectoderm (outer layer), mesoderm (middle layer) and entoderm (inner layer) which is known as the chorioallantois; that inner part of the sac which is not opposed to chorion and which has limited fusion with the amnion is known as the inner wall. This structure serves no significant respiratory function and will not be considered further.

Progressive chorioallantoic growth and fusion results in complete covering of the embryo by six days, half the yolk sac by eight, and then progression to the lower pole of the egg where, by 12 days, it encloses the albumen (98b).

Arising with and within the allantoic stalk are a left and right allantoic (umbilical) artery and their accompany veins. The right allantoic vein atrophies on the fourth day (98b) and the right allantoic artery is of subordinate importance during the last ten days of incubation (128a). Thus, the growing chorioallantoic membrane is supplied by one artery and one vein and these form smaller vessel branches within the mesodermal layer of the chorioallantois. A coarse capillary plexus traverses the mesodermal layer of the allantois by the fourth day and differentiates further with the fusion of the chorion and allantois; extensive arborizations of larger vessels with close, fine arterial and venous interdigitations are visible by the sixth day (128a).

Fülleborn (129) describes the result (translation from reference 98b): "The capillaries form such narrow meshes, and have relatively so wide a lumen, that they can be compared only with those of the lungs of higher animals and of the choroidea of the eye; indeed, instead of describing it as a vascular network embedded in tissue, one could well describe it as a great blood sinus interrupted by strands of tissue." This extensive vascular net is the primary organ for respiratory gas exchange between the developing embryo and the external environment until shortly after the eighteenth day when the respiratory movements start, the beak pierces the inner shell membrane to enter the air space and air breathing begins (128a).

A recent electron microscopic study by Ganote et al. (130) reveals that the mesodermal, or middle, layer of the chorioallantois consists of a loose matrix of fibroblasts and collagen fibrils through which blood and lymphatic vessels course without visible nerve fibers. The inner (entodermal) layer is composed of two layers of imbricated cells interconnected by numerous desmosomes. The outer chorionic layer"... was formed by two rows of ectodermal cells which were connected by numerous desmosomes and interdigitations of plasma membranes. The cells were separated from the mesodermal layer by a basement membrane and from the shell membrane by a narrow space which contained a small amount of amorphous material. At intervals, blood vessels ensheathed by a basement membrane penetrated the

chorionic layer from the mesoderm. Within the chorionic layer they formed an intraepithelial capillary plexus which rested on the basal row of ectodermal cells and tunneled between the cells of the outer row. Thin cytoplasmic processes from the outer row of ectodermal cells arched over the capillaries and separated them from the shell membrane (130)."

The chorioallantoic vessels had been described with the light microscope as progressively penetrating the ectodermal layer until at about 15 days they are free of an ectodermal covering and in direct contact with the inner shell membrane (98b, 128a). However, at no point did Ganote et al., using the electron microscope, observe capillaries actually penetrating the ectoderm. The cytoplasmic processes of the outer ectodermal cells became attenuated over the capillaries and were as thin as the endothelial wall by day 14. Nearly identical results were obtained by the electron microscopic studies of Skalinsky and Kondalenko (131) who observed a membrane 150-200 mμ thick delimiting the vascular lumina from the air space between the fibrils of the inner shell membrane. This membrane was composed of four layers - the endothelial cytoplasm, a basement membrane, the cytoplasm of the chorionic ectoderm and the fringed layer of the inner shell membrane.

Closely applied to chorionic (outer) surface of the chorioallantoic membrane and following the contour of the shell are two shell

membranes (132a). The outer shell membrane lies between the inner shell membrane and the shell and is firmly embedded in the inner surface of the shell (132a). The composite thickness of both egg shell membranes in a Leghorn hen's egg is about 0.065 mm. (132a).

The two shell membranes are tightly apposed and cemented to - gether except in the region of the air space at the blunt end of the egg (132a). Here they first separate after the egg is laid; the outer shell membrane is adherent to the shell and forms the outer wall and the inner shell membrane overlies the chorioallantois, forming the inner wall of the air space. The air space is small early in incubation and probably forms as a result of cooling of the egg contents following laying (133a). This space continues to increase in size with evaporation of egg contents; its volume increases from about 3 cc. at eight days to 7 cc. at 18 days (133a).

The outermost barrier to diffusion into the chorioallantoic capillaries is the egg shell itself. It is a strong protective structure composed of about one part interwoven organic fibers, which are collagen-like, to 50 parts interstitial substance which is a mixture of inorganic salts. Various amounts of water are present between the calcite crystals of the shell (132a). The shell thickness varies considerably with various seasons, flocks and individual hens, but the shell thickness of a Leghorn hen's egg averages about 0.21 mm. (132a). Pene-

trating the total thickness of the shell are minute canals, less than 0.006 mm. in diameter, filled with a matrix of protein fibers, and opening onto the shell surface as larger oval pores. There are slightly over 7500 such pores per hen's egg (132a) and these are unevenly distributed, being most numerous at the blunt end. They are believed to facilitate gas diffusion (132a).

Thus, gases diffusing between capillary blood and the external atmosphere must penetrate a barrier about 0.38 mm. thick composed of: 1. a porous shell, 2. two egg shell membranes, 3. a thin cytoplasmic process of chorionic ectoderm with an overlying basement membrane, and 4. the capillary endothelial wall.

## III. Materials and Methods

### A. Eggs and Incubator

Three experiments, each with 200 eggs, were performed between 4 May 1967 and 16 July 1967. All the experiments were performed by the same person carefully following a recorded experimental procedure (See appendix I). For the first and third experiments, the eggs were incubated in room air; for the second experiment, the eggs were incubated in 60 percent oxygen. The eggs were obtained from a local hatchery where, following laying, they had been stored for 72 to 96 hours in a moist room at 60 degrees Fahrenheit. White Rock (Hubbard strain) eggs from the same flock of hens were used in all three experiments. The eggs were placed in a prewarmed incubator (Forma, Series 66) and were brought to 39.5°C. over a four hour period.

The eggs were held upright in five horizontal trays which automatically tipped through 90 degrees every 3 hours. Heated air was circulated vertically by a fan, but a 1.2° C. temperature gradient existed between the top and bottom trays. Because during previous investigations the incubator heating unit had been found to be sensitive to room temperature, the circuitry was insulated and a constant temperature was maintained around the circuitry. This resulted in an average temperature (in the center of the middle tray) of 39.5° C. with variations of 0.35° C. or less. The incubator temperature was

continuously monitored and recorded (except during the hours of active study) by a shielded glass thermistor located in the center of the middle tray.

The incubator was ventilated with air or 100 percent oxygen at a flow rate of 1050 cc./min. This flow of 100 percent oxygen maintained an incubator atmosphere of 60 percent ±2 percent oxygen. Oxygen concentration was measured with a Beckman D-2 (Pauling type) oxygen analyzer. Incoming gases were not humidified, but the relative humidity (measured by a wet bulb hygrometer) remained between 80 and 85 percent.

B. Preparation of Eggs

Experimental determinations were performed after 8, 10, 12, 14, 16 and 18 days of incubation. On the morning of an experimental day, an appropriate number of eggs were removed from the incubator, a proportionate number from each tray. The eggs were quickly candled to eliminate obviously undeveloped eggs. A round hole 1.5 cm. in diameter was made in the shell overlying the air space of each of the remaining eggs with sharp forceps, thereby opening the airspace to the atmosphere. With candling, the chorioallantoic vessels could easily be visualized. The criterium for a viable egg was spontaneous embryonic movement and a vasculature normal in extent and appearing filled with blood. During these procedures only a few eggs were outside the incubator at one time to minimize cooling. The viable eggs

were replaced in the middle shelf of the incubator and allowed to re-warm for at least two hours before any measurements were performed.

If the eggs to be examined had been incubated in 60 percent oxygen, the incubator was cleared of the high oxygen and supplied with a flow of air from the start of the two hour warming period until that day's experiment was completed in an attempt to reverse any vaso-spasm that might be present as a result of the elevated oxygen; at the conclusion of the day's experiment, the 60 percent oxygen concentration was quickly restored. Thus the eggs remaining in the incubator were exposed to air instead of 60 percent oxygen for about five or six hours on each experimental day.

C. Experimental Equipment and Procedures (See appendix I)

The experiments were designed to measure six parameters: Oxygen consumption, diffusion capacity for carbon monoxide, egg volume, morphological stage of development, embryonic weight and hemoglobin concentration.

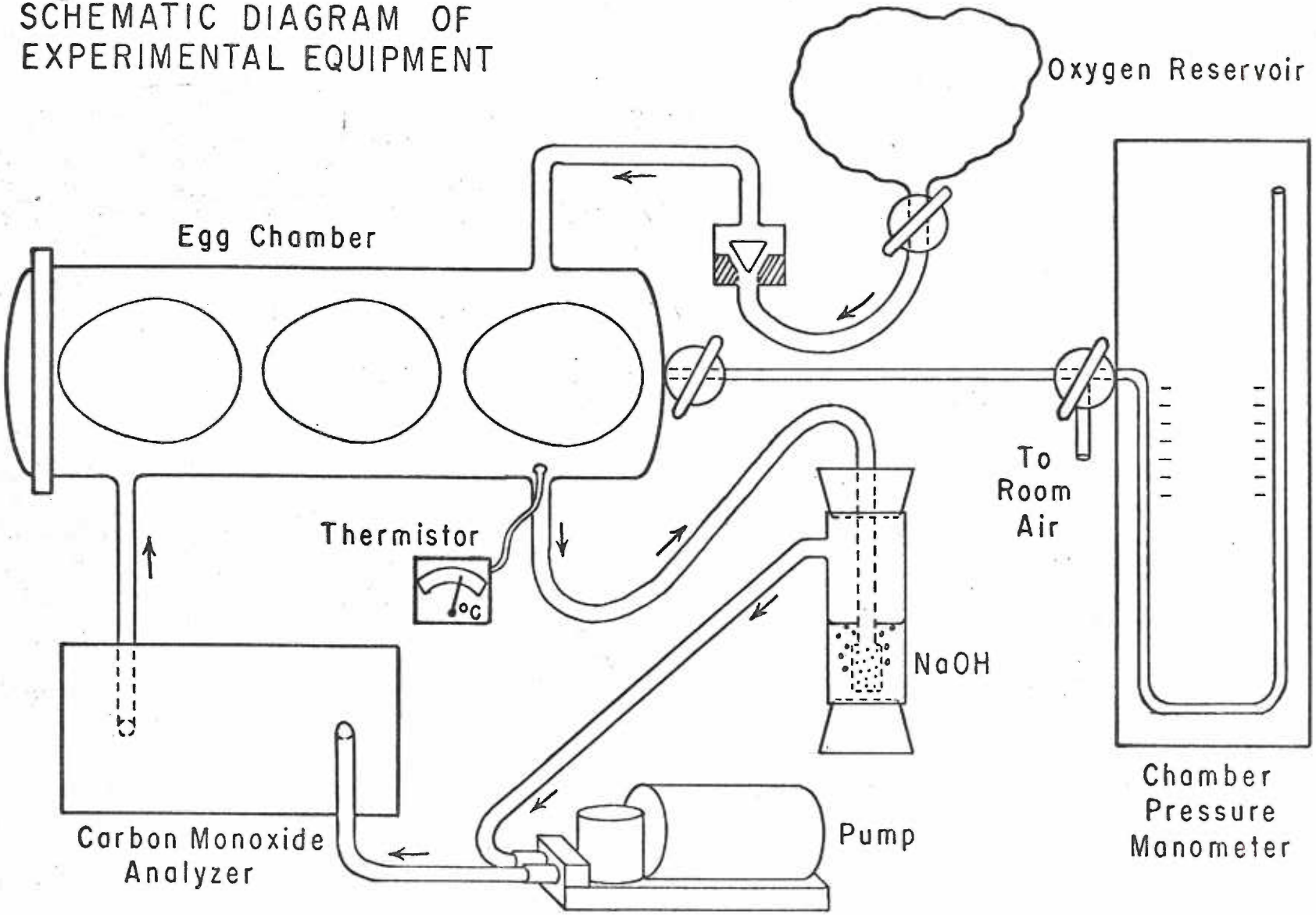
The oxygen consumption and diffusion capacity measurements utilized a closed circulation system (figure 2) composed of four parts: 1. A glass chamber to hold the eggs. 2. A carbon dioxide absorber. 3. A carbon monoxide analyzer. 4. A pump to circulate the gases through these parts.

1. The glass chamber was designed to hold three jumbo sized eggs end to end. It had a removeable air tight door at one end, an

Figure 2

Schematic diagram of experimental equipment for measuring chick embryo Dco and rate of oxygen consumption.

SCHEMATIC DIAGRAM OF
EXPERIMENTAL EQUIPMENT



input tube near one end and an output tube at the other through which the gases circulated. Several other points of access to the chamber were present and each could be sealed by turning a stopcock. One of these was connected to a water manometer to measure pressure changes within the system. Another was connected through a one-way valve to a 400 cc. collapsable Saran bag which served as an oxygen reservoir to maintain a constant $pO_2$ within the system during the measurement of diffusing capacity. A drop in pressure within the chamber of 6-7 mm. $H_2O$ (equivalent to the uptake of 0.25 ml. oxygen) opened the one-way valve and automatically replaced oxygen consumed by the embryos. This gravity loaded valve allowed oxygen to flow into the chamber, but did not allow carbon monoxide to escape.

The eggs rested upon a thick wire mesh floor and were exposed to the circulating gases over essentially all of their shell surfaces. The temperature of the effluent chamber gases was continuously measured with a rapid response, glass tipped thermistor wired to a calibrated indicator.

Once three eggs were in place within the chamber, the chamber door was sealed and the entire chamber submerged in a water bath heated to 39.5° C.

2. The effluent gases from the egg chamber next entered the carbon dioxide absorption chamber. An upright plexiglas tube containing 2 ml. of 8.25 molar sodium hydroxide and a small amount of

silicone antifoam agent (Beckman 104611). A sodium hydroxide solution was chosen over solid sodium hydroxide because the latter is very hydroscopic. The circulating gases were divided into fine bubbles to pass through the sodium hydroxide solution. They exited through the top of the tube to enter the pump.

3. The pump was a closed circuit diaphragm gas pump which was made airtight and circulated the gases through the entire system at about 1300 cc./min. The 90 percent clearing time for an injected volume of carbon monoxide was 15 seconds. The gases exited from the pump and entered the carbon monoxide analyzer.

4. The infrared carbon monoxide analyzer was a Beckman IR 15-A spectrophotometer and amplifier. The spectrophotometer had a sample cell length of 4" which was balanced against a nitrogen-filled cell. The infrared beam was absorbed by five percent carbon dioxide and water vapor to a negligible extent. The output of the spectrophotometer was recorded by a Texas Instruments servo recorder. This had a full-scale response time of slightly over one second.

The gases leaving the spectrophotometer sample cell then reentered the egg chamber, completing the cycle. The total volume of egg chamber, carbon dioxide absorber, pump, spectrophotometer sample cell and the interconnecting (3/13" I.D.) rubber tubing was 520 ml. This system was checked for leaks before each experiment by creating a 30 to 40 cm. $H_2O$ negative pressure inside the circulating system.

A fall of 1 mm./min. or more on the manometer was considered a leak. With a plastic egg in the chamber carbon monoxide concentration fell 1 percent or less over a 15 minute period.

1. Measurement of Oxygen Consumption

Oxygen consumption was measured with a water manometer connected through fine tubing (to minimize volume) to the egg chamber. The manometer was constructed of similar tubing in a U-shape and contained water with a drop of green ink to facilitate reading and a few grains of detergent to reduce surface tension. One end was open to the atmosphere. The tubing was set against a white board with millimeter markings by which differences in height of the columns could be accurately measured.

By withdrawing measured amounts of air from the system and noting the resultant pressure drop a value for mm. $H_2O\Delta P$/ml. gas removed was obtained with a standard deviation on successive trials of $\pm 1.0\%$. Because the gas volume varied inversely with the total volume of the three eggs being studied, this ratio (mm. $H_2O\,\Delta P$/ml. gas) depended upon the 3-egg volume. Assuming that all the $CO_2$ produced by the eggs was absorbed, and knowing this ratio for a given egg volume, the $\Delta P_{H_2O}$ in a known time period was entirely due to oxygen consumption which could be calculated per chick per unit time.

The eggs were kept in the incubator until immediately before use; then three eggs were removed, quickly candled to insure viability

and promptly placed into the prewarmed egg chamber. The chamber door was sealed and the chamber was lowered into the water bath. For one minute the circulating gases were allowed to expand into the external atmosphere as their temperature approached 39.5° C. (a minimal standard amount of expansion occurred after this time period, and taken into account in calculating oxygen consumption). At the end of one minute the system was closed to the external environment and the oxygen reservoir was closed to the egg chamber. Oxygen consumption was measured for precisely five minutes.

2. Measurement of Diffusion Capacity

Following the measurement of oxygen consumption, the deficit in oxygen was replenished by opening the oxygen reservoir. The entrance to the manometer was then closed to prevent subsequent loss of carbon monoxide. A precision 0.25 ml. syringe was flushed with 100 percent carbon monoxide for five seconds and then, by use of a standard plunger stop, 0.22 cc. of the gas was retained in the syringe and injected promptly into the circulating system by way of a needle inserted through rubber tubing covered with 3 mm. silastic sealer. The standard deviation of ten successive injections was $\pm 0.6\%$ of the total reading.

The uptake of carbon monoxide by the chick embryos was continuously recorded as the relative decrease of carbon monoxide concentration per unit time; from this and knowledge of the volume of the

three eggs being studied, the diffusion capacity was calculated (See appendix III).

3. Measurement of Egg Volume

The egg volume was determined by displacement of saline. An egg was placed into a beaker with fixed overflow volume and the beaker was filled to overflowing (the opened air space was filled also); the egg was removed, the air space volume left in the beaker, and the volume of saline in the beaker was measured with a graduated cylinder. Egg volumes were consistently reproducible to ± 1cc.

4. Measurement of Stage of Development of Embryos

The morphological development of each embryo was measured according to the criteria of Hamburger and Hamilton (98c). Readily obtainable and quantifiable criteria were used for each chick. An age equivalent was not recorded at the time of evaluation of the embryo to minimize judgemental bias (See appendix II).

5. Measurement of Weights

The net weight of each chick was obtained by cutting the yolk and allantoic stalks at their entrance into the abdomen, and excising the amnion. The embryo was gently washed with saline to free it of yolk and of membranes, allowed to drip free of excess moisture and weighed to the nearest 0.1 gm. upon a Harvard balance.

6. Measurement of Hemoglobin Concentration

Chorioallantoic blood was obtained after candling the egg to

identify a suitable vessel. With the aid of a metal file (such as are supplied to open glass ampules) a 1 $cm^2$. area of shell and its outer shell membrane was removed. The inner shell membrane was rendered translucent with a drop of mineral oil and the chorioallantoic vessels were then easily visualized. A 25 gauge needle tip attached to a 10" length of polyethylene catheter was placed within the vessel and about 50 µl. of blood was withdrawn by gentle suction upon the catheter. Precisely 20 µl of blood was placed into a premeasured cyanide solution (Unopette) and the resulting solution was read for cyanmethemoglobin concentration at 540 mµ. against a standard hemoglobin solution using a Coleman Junior spectrophotometer.

The results at all ages within each experimental group were considered as one sequential result for that group and these overall group means were compared for statistically significant differences by the method of least squares factorial analysis for unequal cell frequencies and significant differences between individual age groups were determined by the Newman-Keuls method, according to Winer (134).

# IV. Results

## A. Egg Volumes

Average 3-egg volumes were : Room Air #1 - 192 ml., 60 percent $O_2$ - 190 ml., Room Air #2 - 183 ml.

## B. Egg Viability

The following percentage of eggs were judged viable by examination at the time they were being studied: Room Air #1 - 55%, 60 percent $O_2$ - 57%, Room Air #2 - 66%.

## C. Weights

The weights of chick embryos followed a rising curve of increasing steepness with age (figure 3). No significant difference occurred between the overall means of the two room air groups (Table 1) and they were combined to form the "pooled room air" eggs. The variances are relatively small at each age and the average "pooled room air" embryo at each age is significantly ($p < 0.01$) heavier than the average embryo of the preceding age.

The difference between the weights of the "pooled room air" embryos and the 60 percent oxygen embryos is significant ($p < 0.01$); and the more rapid increase in weight with age in 60 percent oxygen embryos is indicated by the significant interaction effect between ages and conditions ($p < 0.01$).

## D. Stage

The morphological stage of embryonic development increases

Figure 3

Graph of chick embryo weight vs. age.

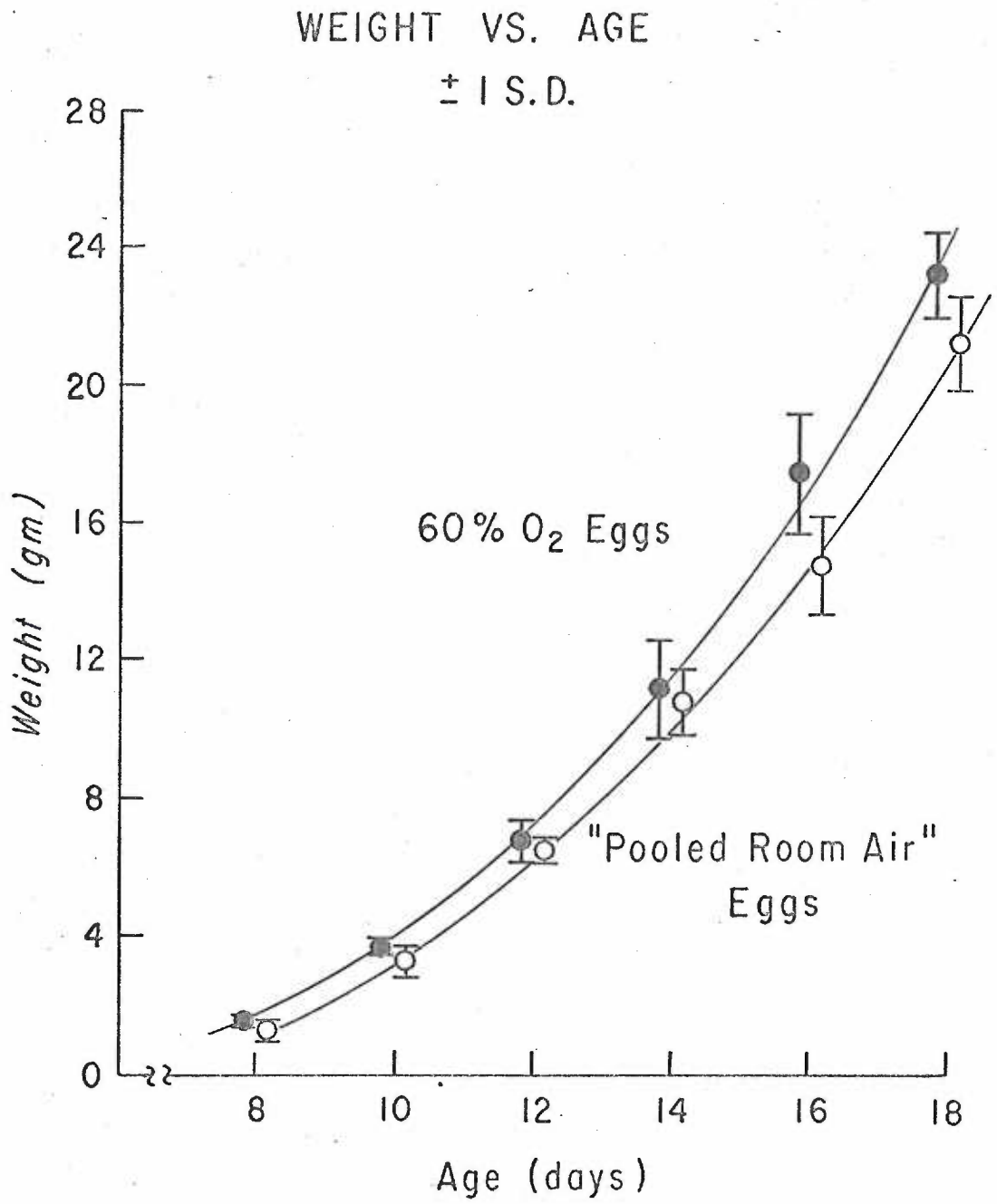

WEIGHT VS. AGE
± 1 S.D.
28
24
20
16
12
8
4
0
Weight (gm)
60% $O_2$ Eggs
"Pooled Room Air" Eggs
8
10
12
14
16
18
Age (days)

Table I

Weight (gms.)

| Group | | Days of Incubation 8 | 10 | 12 | 14 | 16 | 18 | p** |
|---|---|---|---|---|---|---|---|---|
| First | $\bar{X}$ | 1.3 | 3.0 | 6.2 | 10.3 | 14.6 | 21.6 | Conds. |
| Room | S.D. | 0.1 | 0.2 | 0.2 | 0.6 | 1.4 | 1.2 | $>0.25$ |
| Air | N | (6) | (7) | (6) | (7) | (6) | (7) | |
| Second | | 1.2 | 3.5 | 6.8 | 11.3 | 14.9 | 20.8 | Ages x |
| Room | | 0.2 | 0.2 | 0.5 | 1.0 | 1.3 | 1.5 | Conds. |
| Air | | (6) | (7) | (6) | (7) | (6) | (9) | $>0.25$ |
| Pooled | | 1.3 | 3.3 | 6.5 | 10.8 | 14.8 | 21.2 | Conds. |
| Room | | 0.2 * | 0.4 * | 0.4 * | 1.0 * | 1.4 * | 1.5 | $<<0.01$ |
| Air | | (12) | (14) | (12) | (14) | (12) | (16) | |
| Sixty | | 1.6 | 3.7 | 6.8 | 11.2 | 17.5 | 23.2 | Ages x |
| Percent | | 0.0 | 0.2 | 0.7 | 1.3 | 1.7 | 1.3 | Conds. |
| Oxygen | | (6) | (6) | (4) | (6) | (6) | (6) | $<0.01$ |

* $p<0.01$ between these two cells

** In the above table and following tables the heading "p" for "conditions" refers to the statistical comparison between the means of the two control room air groups, and to the statistical comparison between the means of the pooled room air and 60% $O_2$ groups. The designation "ages x conditions" identifies the statistical interaction between ages and conditions for the first and second room air groups, and for the pooled room air and 60% $O_2$ groups.

linearly between days 8 and 18 (figure 4). There was no significant difference in the data of the two room air groups (Table II) and they were combined as "pooled room air" eggs. At each age, the average "pooled room air" chick was significantly more developed than the "pooled room air" chick of the preceding age.

The embryos incubated in 60 percent oxygen were slightly further developed at each age than room air embryos of similar age and the difference is significant ($p < 0.01$) when the overall means of the two groups are compared.

E. Hemoglobin

Hemoglobin samples were obtained only from the second room air group of embryos (Table III). The blood hemoglobin concentration increased with age (figure 5). The relatively large variances at each age limited the significant differences between ages to those between 14 and 16 days ($p < 0.05$).

F. Oxygen Consumption

Figure 6 indicates the inverse relationship between oxygen consumption per gram of tissue per hour, and age. Again, no significant difference was found between the two groups of data from room air eggs (Table IV) and they were combined as "pooled room air" eggs. The oxygen consumption of the "pooled room air eggs" was consistently higher than that of eggs incubated in 60 percent oxygen ($p \ll 0.01$) when oxygen consumption was measured in a room-air environment.

Figure 4

Graph of chick embryo morphological stage (according to Hamburger and Hamilton (98c)) vs. age (length of incubation).

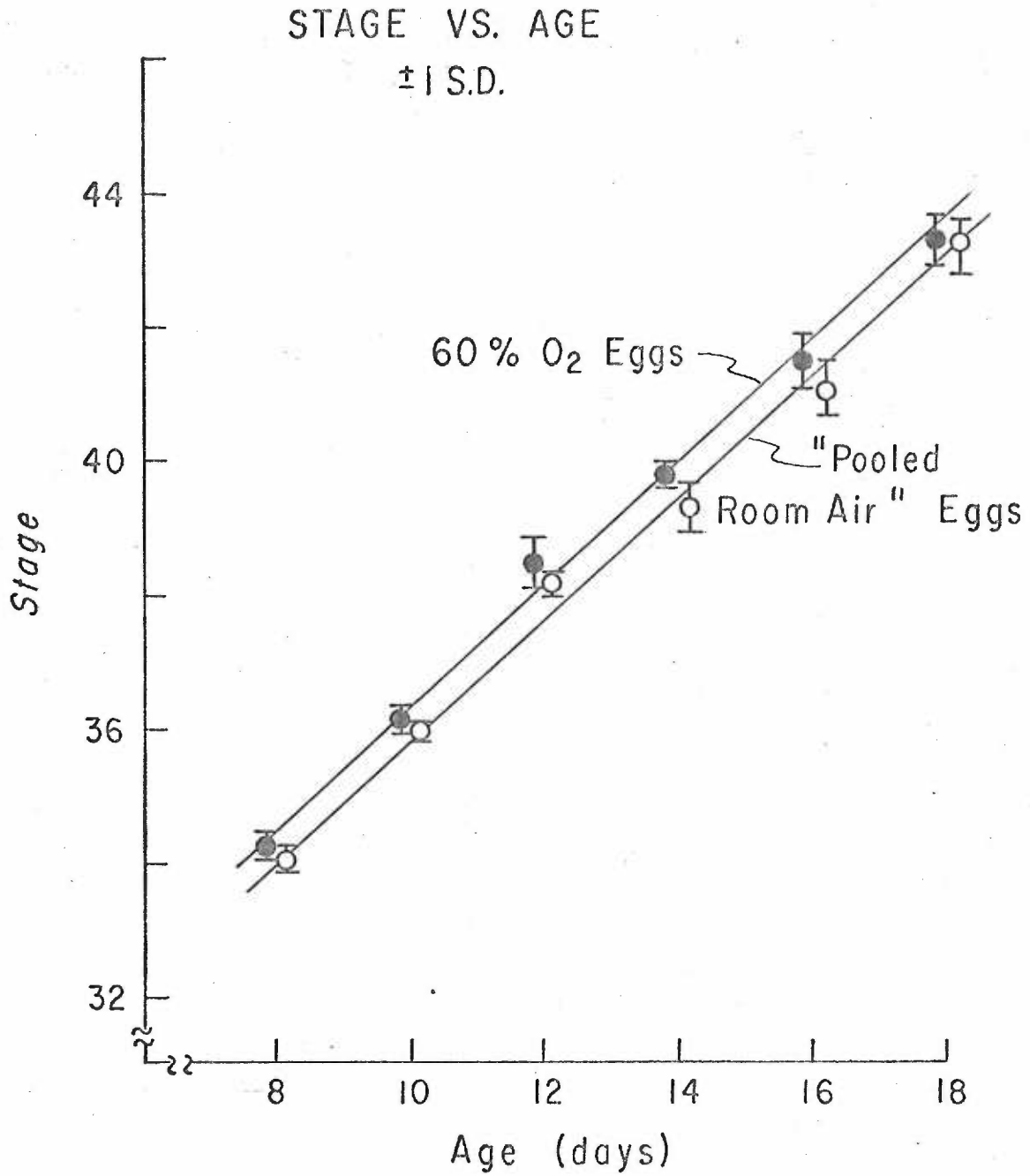

STAGE VS. AGE
±1 S.D.
44
40
36
32
Stage
60 % $O_2$ Eggs
"Pooled
Room Air" Eggs
8
10
12
14
16
18
Age (days)

Table II

Stage

| Group | | Days of Incubation 8 | 10 | 12 | 14 | 16 | 18 | p |
|---|---|---|---|---|---|---|---|---|
| First | $\bar{X}$ | 34.0 | 36.0 | 38.3 | 39.4 | 41.0 | 43.1 | Conds |
| Room | S.D. | 0.0 | 0.0 | 0.4 | 0.4 | 0.5 | 0.4 | $>0.25$ |
| Air | N | (6) | (7) | (7) | (7) | (6) | (7) | |
| Second | | 34.2 | 36.1 | 38.1 | 39.3 | 41.2 | 43.3 | Ages x |
| Room | | 0.2 | 0.2 | 0.2 | 0.4 | 0.4 | 0.5 | Conds |
| Air | | (6) | (7) | (6) | (7) | (6) | (9) | $>0.25$ |
| Pooled | | 34.1 | 36.0 | 38.2 | 39.3 | 41.1 | 43.2 | Conds |
| Room | | 0.2 * | 0.1 * | 0.2 * | 0.4 * | 0.4 * | 0.4 | $<<0.01$ |
| Air | | (12) | (14) | (13) | (14) | (12) | (16) | |
| Sixty | | 34.3 | 36.2 | 38.5 | 39.8 | 41.5 | 43.3 | Ages x |
| Percent | | 0.2 | 0.2 | 0.4 | 0.2 | 0.4 | 0.4 | Conds |
| Oxygen | | (6) | (6) | (7) | (6) | (6) | (6) | $>0.25$ |

* $p<0.01$ between these two cells.

Table III

Hemoglobin Concentration in
Second Room Air Chicks
(gms. /100 ml. blood)

| | Days of Incubation | | | | | |
|---|---|---|---|---|---|---|
| | 8 | 10 | 12 | 14 | 16 | 18 |
| | 5.1 | 5.8 | 5.4 | 6.6 | 8.1 | 9.1 |
| S.D. | ±1.0 | ±0.4 | ±0.7 | ±0.8 | ±0.9 | ±1.1 |
| N | 5 | 7 | 4 | 7 | 4 | 6 |

Figure 5

Graph of chick embryo hemoglobin concentration vs. age (second room air group only). Chick embryo hemoglobin concentrations obtained by Barnes, A.E. and Jensen, W.N. (Am. J. Physiol. 197:403-405, 1959) are included for comparison.

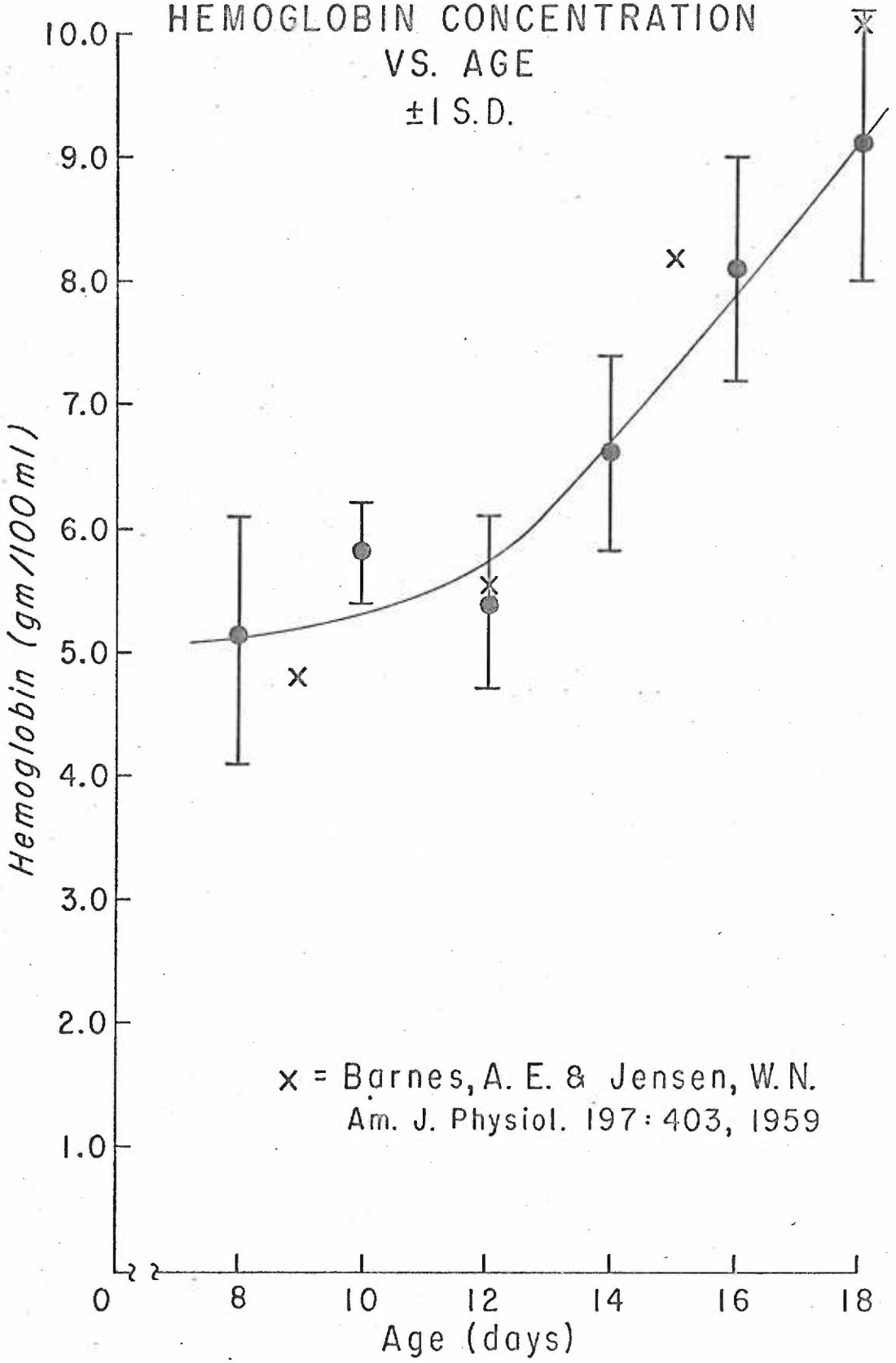

HEMOGLOBIN CONCENTRATION
VS. AGE
±1 S.D.
Hemoglobin (gm/100 ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
8
10
12
14
16
18
Age (days)
x = Barnes, A. E. & Jensen, W. N.
Am. J. Physiol. 197: 403, 1959

Figure 6

Graph of rate of oxygen consumption per gram vs. age. Averages of values obtained in chick embryos by Hasselbalch and Murray, as tabulated by Needham, J. (Chemical Embryology, Hafner, N. Y. 1963, p. 698 (Fig. 143)) are included for comparison.

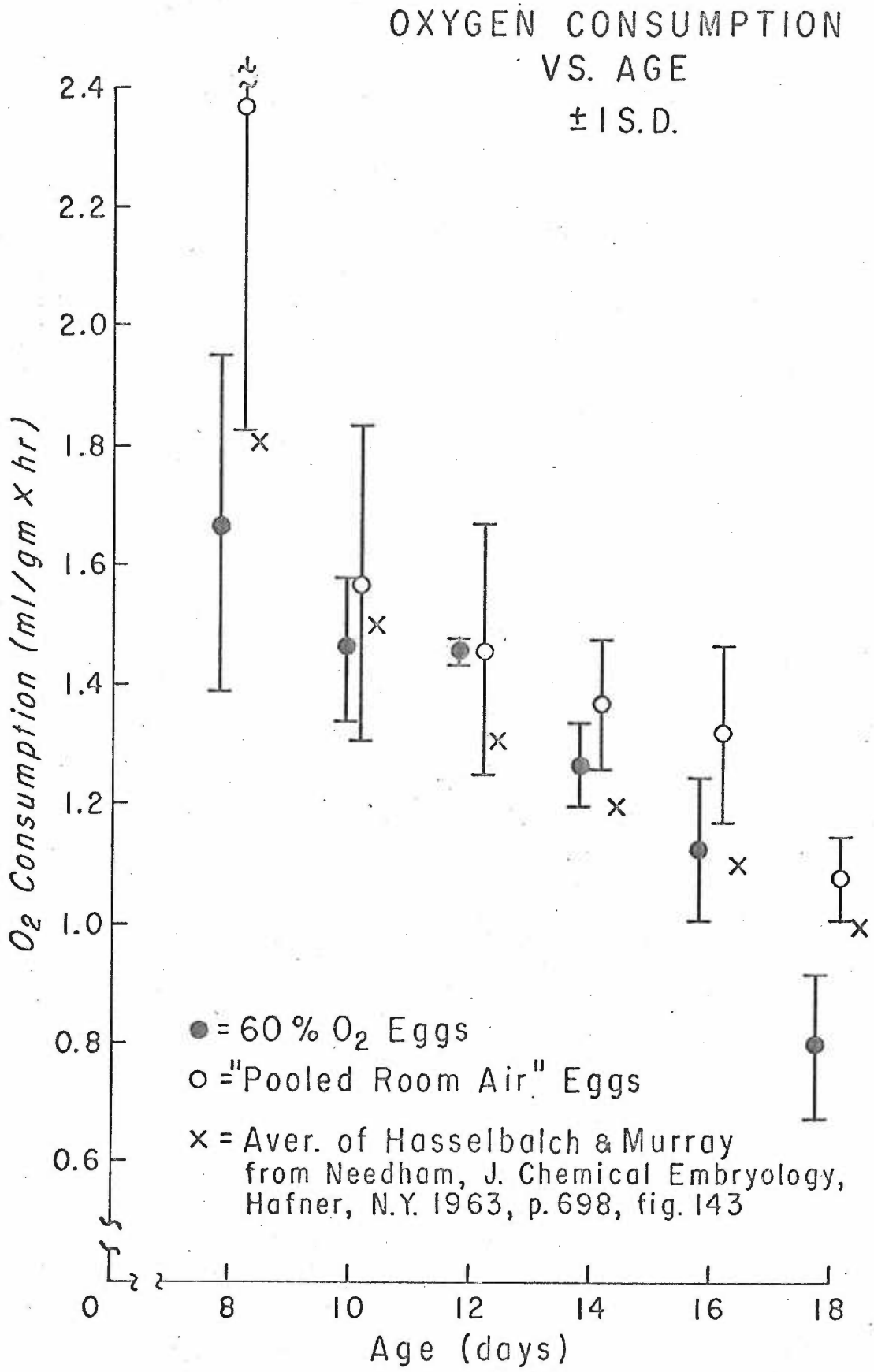

OXYGEN CONSUMPTION
VS. AGE
±1 S.D.
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0
O2 Consumption (ml/gm x hr)
● = 60 % O2 Eggs
○ = "Pooled Room Air" Eggs
× = Aver. of Hasselbalch & Murray
from Needham, J. Chemical Embryology,
Hafner, N.Y. 1963, p. 698, fig. 143
8
10
12
14
16
18
Age (days)

Table IV

Oxygen Consumption
(ml./gm. x hr.)

| Group | | Days of Incubation 8 | 10 | 12 | 14 | 16 | 18 | p |
|---|---|---|---|---|---|---|---|---|
| First | $\bar{X}$ | 2.02 | 1.66 | 1.59 | 1.41 | 1.36 | 1.12 | Conds |
| Room | S.D. | 0.80 | 0.31 | 0.08 | 0.12 | 0.20 | 0.04 | $>0.10$ |
| Air | N | (6) | (6) | (7) | (7) | (6) | (7) | |
| Second | | 2.72 | 1.50 | 1.30 | 1.34 | 1.28 | 1.05 | Ages x |
| Room | | 0.47 | 0.24 | 0.21 | 0.10 | 0.04 | 0.08 | Conds |
| Air | | (6) | (7) | (6) | (7) | (6) | (9) | $>0.05$ |
| Pooled | | 2.37 | 1.57 | 1.46 | 1.37 | 1.32 | 1.08 | Conds |
| Room | | 0.76 * | 0.26 | 0.21 | 0.11 | 0.15 ** | 0.07 | $<<0.01$ |
| Air | | (12) | (13) | (13) | (14) | (12) | (16) | |
| Sixty | | 1.67 | 1.46 | 1.46 | 1.27 | 1.13 | 1.802 | Ages x |
| Percent | | 0.28 | 0.12 | 0.02 | 0.07 | 0.12 | 0.121 | Conds |
| Oxygen | | (6) | (6) | (4) | (6) | (6) | (5) | $<0.05$ |

* $p<0.01$ between these two cells.
** $p<0.05$ between these two cells

At 8 days, the total volume of oxygen consumed by three eggs in five minutes was less than 0.4 cc., which is about the minimum volume this method can accurately measure and, consequently, there was a large variance in individual results at this age. The value of 2.37 ml./gm./hr. obtained for 8 day old room air chicks is about three standard deviations higher than would be predicted from the pattern of the remaining data and may be erroneously high. A rather large variance occured in the remaining age groups as well and, in "pooled room air" eggs, significant differences between adjacent age groups occured only between 8 and 10 days ($p<0.01$) and 16 and 18 days ($p<0.05$). The analysis of variance indicates a significant interaction between the experimental variables, ages and conditions (ambient $pO_2$) the explanation of which is not apparent (figure 5).

G. Dco

The diffusion capacity for carbon monoxide increased in fertile eggs during incubation. The graph of Dco against time of incubation approximates a sigmoid curve (figure 7) with a decreasing upward slope at 14 to 18 days. As indicated in Table V, there was no significant difference between the mean values of the first and second room air groups and their data were combined as "pooled room air" eggs. The value of Dco at each age was significantly different from values at all other ages studied ($p<0.01$) except between 16 and 18 day results ($p>0.05$) in the "pooled room air" eggs.

Figure 7

Graph of Dco vs. age.

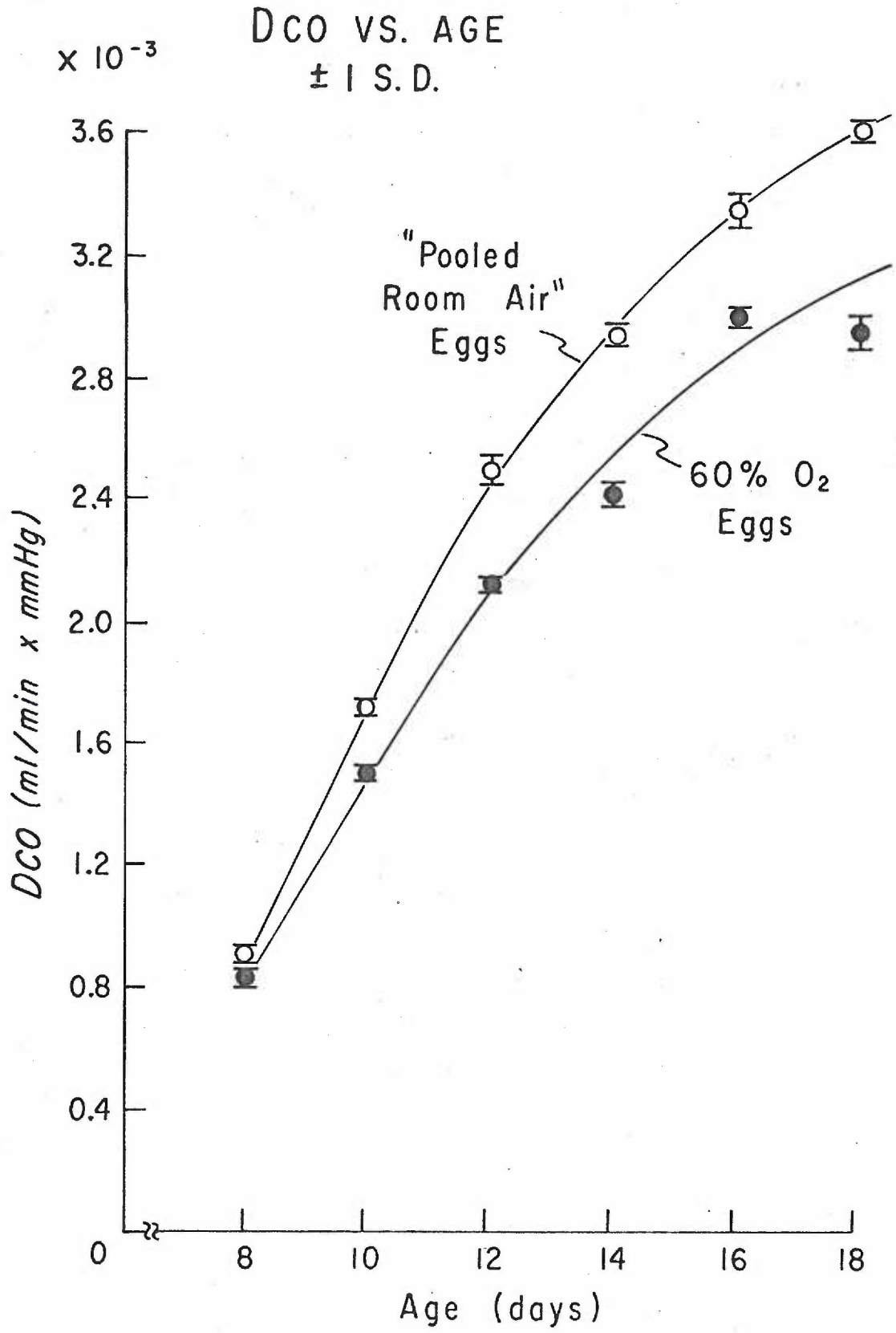

DCO VS. AGE
± 1 S.D.
x 10⁻³
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
DCO (ml/min x mmHg)
"Pooled Room Air" Eggs
60% O2 Eggs
8
10
12
14
16
18
Age (days)

Table V

Dco (ml. /min. x mm. Hg.) x $10^{-3}$

| Group | | Days of Incubation | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 8 | 10 | 12 | 14 | 16 | 18 | p |
| First | $\bar{X}$ | 1.00 | 1.81 | 2.59 | 2.85 | 3.34 | 3.62 | Conds |
| Room | S.D. | 0.36 | 0.32 | 0.46 | 0.19 | 0.61 | 0.26 | > 0.25 |
| Air | N | (6) | (7) | (7) | (7) | (6) | (7) | |
| Second | | 0.84 | 1.62 | 2.40 | 3.00 | 3.38 | 3.56 | Ages x |
| Room | | 0.22 | 0.18 | 0.60 | 0.36 | 0.35 | 0.37 | Conds |
| Air | | (6) | (7) | (6) | (7) | (6) | (9) | > 0.25 |
| Pooled | | 0.91 | 1.72 | 2.50 | 2.94 | 3.35 | 3.59 | Conds |
| Room | | 0.30 * | 0.28 * | 0.51 * | 0.30 * | 0.50 | 0.33 | << 0.01 |
| Air | | (12) | (14) | (13) | (14) | (12) | (16) | |
| Sixty | | 0.84 | 1.51 | 2.12 | 2.42 | 3.00 | 2.95 | Ages x |
| Percent | | 0.18 | 0.22 | 0.19 | 0.44 | 0.29 | 0.61 | Conds |
| Oxygen | | (6) | (6) | (4) | (6) | (6) | (5) | > 0.25 |

* $p < 0.01$ between these two cells

A comparison of the room air data with those from eggs incubated in 60 percent oxygen shows a significantly lower Dco for the eggs incubated in 60 percent oxygen ($p<0.01$). The difference in Dco between these two groups increases with increasing length of incubation.

# V. Discussion

## A. Evaluation of Methods

In order to assess the accuracy of a method it is convenient to have data obtained by other investigators using different methods. No such data are available for the diffusion capacity but several measurements of chick oxygen consumption have been reported. The results of Hasselbalch and Murray, as tabulated by Needham (133b), have been averaged and plotted in figure 6. There appears to be fairly close agreement in terms of range and slope between the published values and the results obtained in this experiment. A similar comparison has been made with values of chick embryo hemoglobin as obtained by Barnes and Jensen (135) and the agreement with data obtained in this study is good.

The percentage of viable eggs at the time of the experiments is low. However, the hens in this flock were old and the hatchery from which the eggs were obtained experienced hatchabilities for the same flock during the same period which were only one to four percent higher than the percentage viable in this experiment. However, the difference in viability was probably greater than four percent because a high chick embryo mortality occurs around the time of hatching and none of the eggs in this experiment were allowed to hatch.

In order to provide a control for effects due to aging, seasons, etc., one room air group preceded and another followed the 60 percent

oxygen group. In none of the measured parameters was there a significant difference between the two room air groups.

Several factors may have influenced the accuracy of the results but should have been equally important in each of the three groups. On this basis, they would not account for differences between room air and 60 percent oxygen chicks. The most important of these is the rise in pCO in the blood of the chick embryo and the percentage saturation of hemoglobin with carbon monoxide during measurements of Dco. The measurement of Dco by the present method requires the assumption that the pCO of the blood entering the chorioallantoic capillaries is negligible. With this assumption the amount of diffusion from the capillary blood outward is insignificant (See appendix II) and the concentration gradient for carbon monoxide diffusion is equal to the concentration of carbon monoxide in the gases surrounding the egg. Therefore, the rate of fall of carbon monoxide concentration external to the egg is proportional to the carbon monoxide concentration external to the egg, i.e. $dc/dt=kC$ outside, where k is a constant. The decay of carbon monoxide concentration in the surrounding gases will then be a first order exponential and a plot of log concentration against time will be a straight line. When the blood concentration of carbon monoxide has risen significantly, this assumption is no longer valid and the rate of fall of carbon monoxide external to the egg becomes $dc/dt=k\ (Cout-Cin)$. This experiment made no provision to measure

Cin; however, when Cin becomes important the plot of log concentration of carbon monoxide external to the egg (Co) against time will become curvilinear with a gradually decreasing slope. Thus the shape of carbon monoxide decay provides a means of testing the assumption of a negligible pCO in chick blood during measurements of Dco. Indeed, examination of eight and ten day chick carbon monoxide decay curves (figure 8) reveals a straight line plot for the first four to six minutes with gradually decreasing curvilinear decay thereafter. A curvilinear decay is not apparent within the first eight minutes with 14, 16 or 18 day eggs (See figure 10).

Another test of this assumption is demonstrated in figure 9, which indicates the calculated percentage of total hemoglobin saturated with carbon monoxide and the pCO in/pCO out ratios of chick embryo blood after three and one-half minutes of exposure to CO at each age. These calculations are based upon the average percent carbon monoxide decay at three and one-half minutes at each age and assume that this percentage of the 0.22 ml. of carbon monoxide injected resides completely within the blood of the embryo. Hemoglobin concentrations at each age were taken from figure 5 and blood volumes were obtained from a plot of the blood volumes of chick embryos at different ages as determined by Barnes and Jensen(135), which agree fairly closely with values given by Romanoff (128b). From these the total circulating hemoglobin was calculated and the percent hemoglobin sat-

Figure 8

Rates of uptake of CO by 8 and 10 day eggs incubated in room air or 60 percent oxygen. These single examples were randomly picked from representative Dco measurements of 8 and 10 day eggs incubated in room air or 60 percent oxygen. These graphs show an initial linear decay of CO followed by a nonlinear decay which becomes evident at about four to six minutes. (All eggs had a hole in the air space shell.)

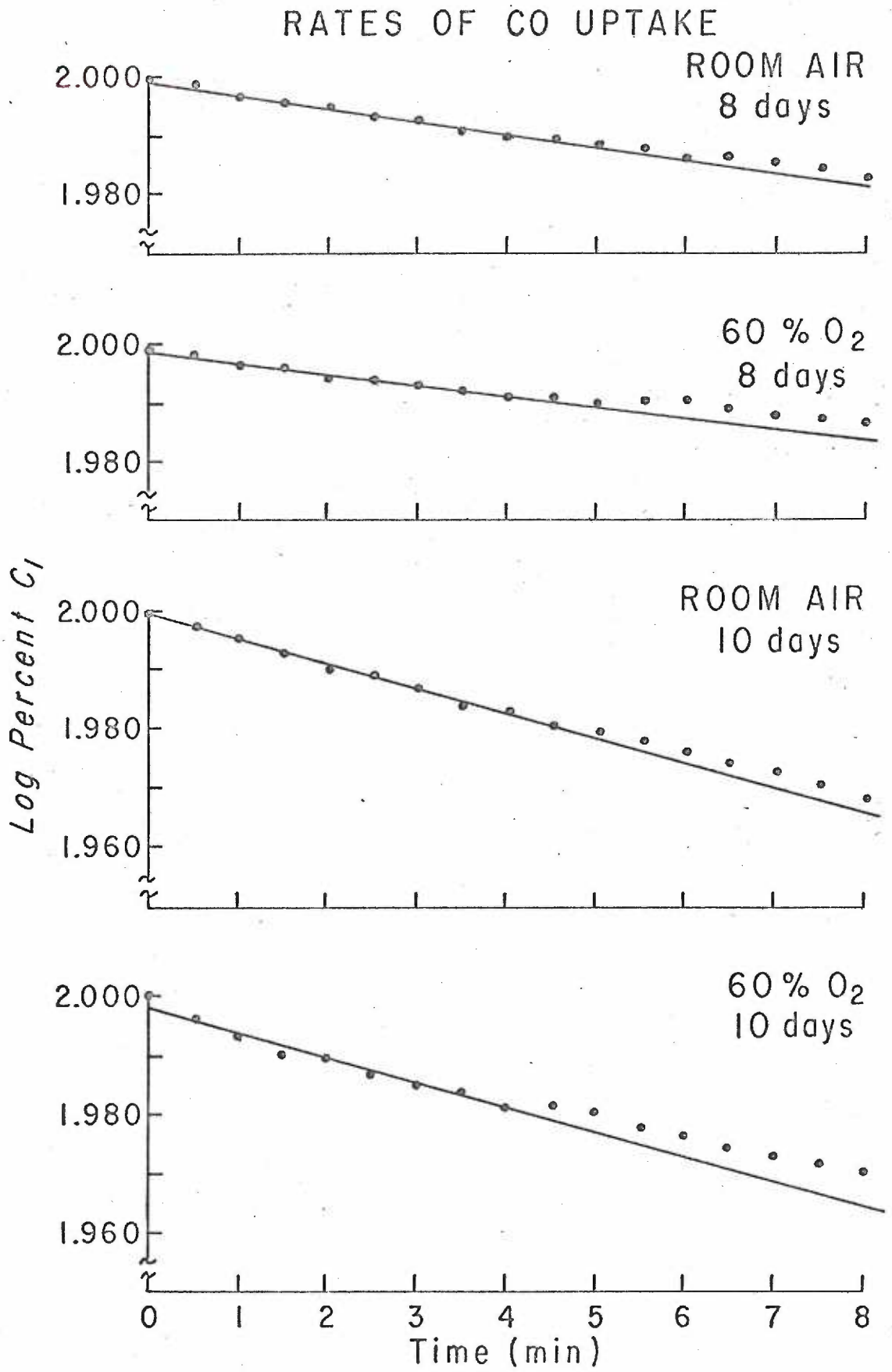
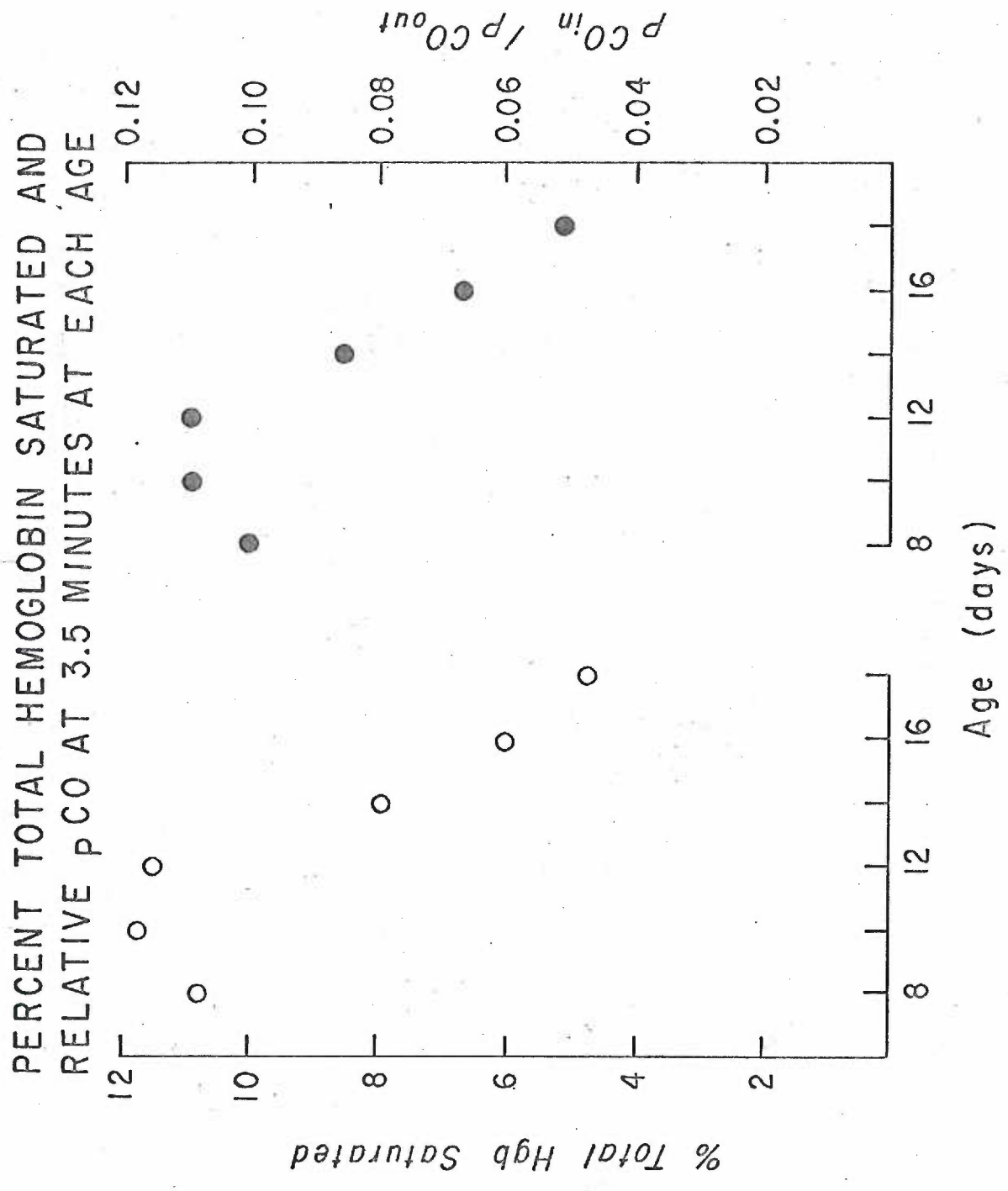

RATES OF CO UPTAKE
ROOM AIR
8 days
60 % O2
8 days
ROOM AIR
10 days
60 % O2
10 days
Log Percent C1
2.000
1.980
1.960
0
1
2
3
4
5
6
7
8
Time (min)

Figure 9

Graphs of the calculated percent total hemoglobin saturated with CO and of the relative pCO at 3 1/2 minutes during the measurement of Dco in room air chicks. Average percents of CO uptake at 3 1/2 minutes were 1.7, 3.8, 5.8, 6.2, 7.3 and 7.7 at 8, 10, 12, 14, 16 and 18 days respectively. Blood volumes (interpolated from the data of Barnes and Jensen (135)) were 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mls. at 8, 10, 12, 14, 16 and 18 days respectively. See text for further details.


PERCENT TOTAL HEMOGLOBIN SATURATED AND
RELATIVE pCO AT 3.5 MINUTES AT EACH AGE
% Total Hgb Saturated
12
10
8
6
4
2
0.12
0.10
0.08
0.06
0.04
0.02
$P_{CO_{in}} / P_{CO_{out}}$
8
12
16
Age (days)

urated with carbon monoxide was obtained from the relationship that one gram of hemoglobin binds 1.34 ml. carbon monoxide. The ratio pCO in/ pCO out was calculated from a normally shaped hemoglobin oxygen dissociation curve (136a) with a 50 percent saturation at a $pO_2$ of about 30 mm. Hg. (as determined by Bartels et al. (137), in the 17 day old chick embryo) assuming a hemoglobin affinity for carbon monoxide 210 times that for oxygen (136b).

Figure 9 shows that for eight, ten and twelve day embryos an average of 11-12 percent of the total hemoglobin had been saturated by the end of the Dco measurement period of 3 1/2 minutes. Similarly, the pCO in/pCO out ratios at these ages were 0.10 to 0.11. These calculations are quite indirect and might be misleading, but even if they are reduced by one-half they still suggest that the blood pCO is not negligible at 3 1/2 minutes. The concentration gradient for diffusion of carbon monoxide at 3 1/2 minutes at eight to twelve days of age may actually be about ten percent less than was assumed. The error would be less at older ages. However, non-linearity during the first 3 1/2 minutes at any age is not evident by visual inspection of the decay plots at any age.

Another factor which may have influenced the results is opening the air space two to five hours before measurement of oxygen consumption and Dco. Earlier observations using eggs with intact shells had shown that the linear decrease of log % $C_1$ vs. time was initiated by a

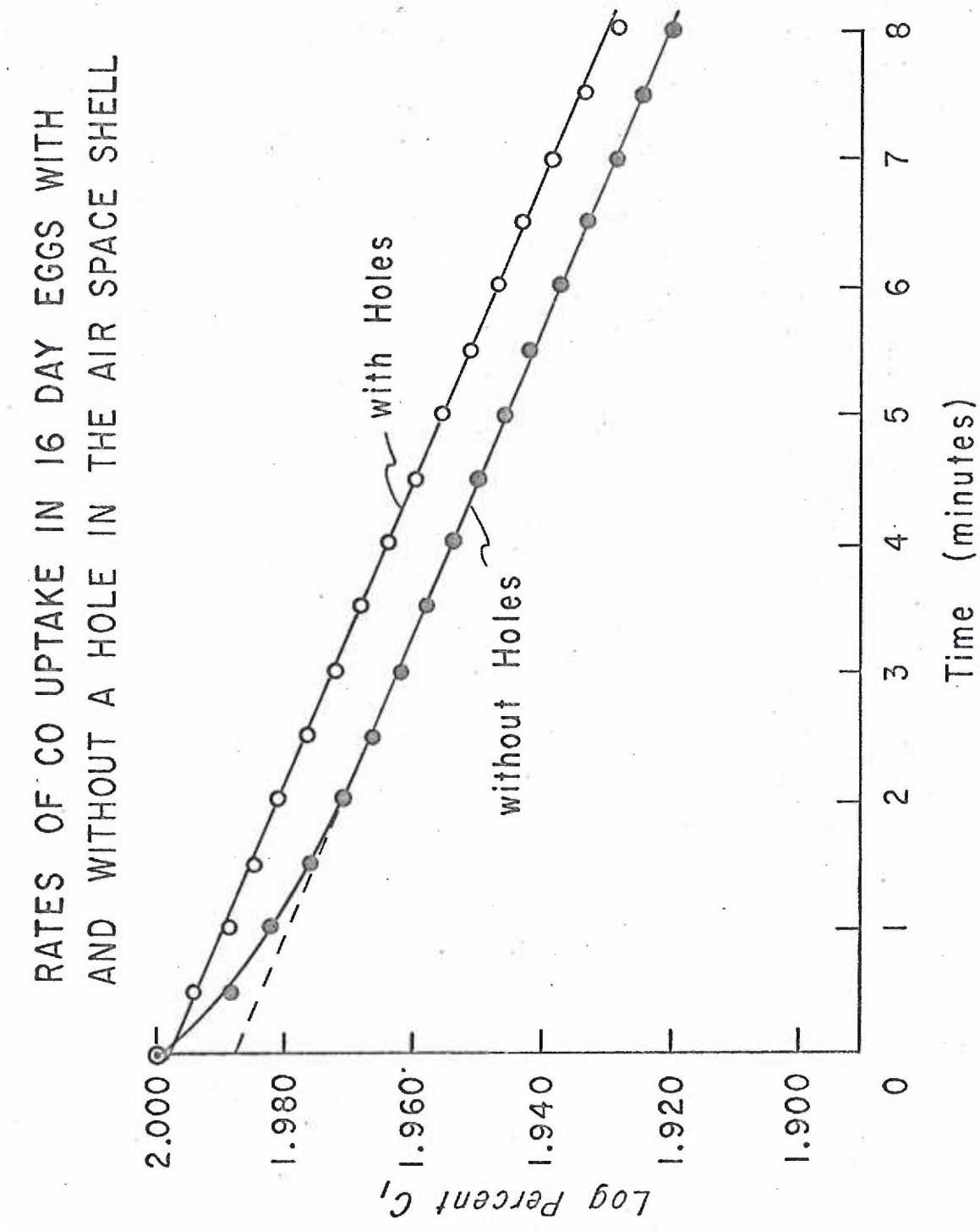
steep non-linear curve for about the first one-half to two minutes in nearly all eggs, and older eggs had a steeper and more prolonged initial non-linearity. Eggs with open air spaces did not show this non-linearity (figure 10) and it was concluded that the initial rapid fall of carbon monoxide concentration had been due to a much more rapid diffusion into the air space than into the rest of the egg. Because this relatively fast diffusion into the air space erroneously steepened the curve from which the Dco was calculated and because it was impractical to use a later part of the curve for this calculation due to the concurrent elevation of blood pCO, it was decided that an open air space would give the most accurate measurement of Dco for the whole egg.

Two hazards are incurred by this procedure. One is drying of the membranes forming the inner wall of the air space because the shell is important in minimizing evaporation (132b); the other is removing a part of the natural barrier to diffusion between the atmosphere and the blood and thereby interfering with measurements of oxygen consumption and diffusion capacity. The importance of the first is uncertain and no attempt was made to measure the amount of drying of these membranes during the two to five hour warming period prior to the experiment nor the effects of this upon the embryo. The second hazard is evaluated in figure 10 which presents the results of an earlier experiment comparing the rates of carbon monoxide uptake

Figure 10

Graphs of average rates of CO uptake in 16 day eggs with and without holes in the air space shell. This experiment was performed on 17 March 1967 with eggs of the same clutch. Holes were placed in the shell overlying the air space in about one-half the eggs 2 to 4 hours prior to measurement of Dco.

This graph demonstrates (1) the initial rapid nonlinear uptake of CO by intact eggs which is absent in eggs with holes, (2) that 16 day embryos have sufficient total hemoglobin that a nonlinear decay, evidence of a significant degree of hemoglobin saturation with CO, is not apparent even at eight minutes, and (3) that the hole in the air space shell does not importantly alter the Dco. Calculations of Dco between 3 1/2 and 7 minutes reveal an average Dco of $3.2 \times 10^{-3}$ ml./min. x mm. Hg. for eggs with holes and an average Dco of $3.0 \times 10^{-3}$ ml./min. x mm. Hg. for eggs without holes.


RATES OF CO UPTAKE IN 16 DAY EGGS WITH
AND WITHOUT A HOLE IN THE AIR SPACE SHELL
with Holes
without Holes
Log Percent $C_1$
2.000
1.980
1.960
1.940
1.920
1.900
0
1
2
3
4
5
6
7
8
Time (minutes)

in 16 day eggs with and without holes in the shell overlying the air space. It is visually apparent that between 3 and 8 minutes the two groups have approximately the same Dco; calculations of Dco between 3 1/2 and 7 minutes reveal a slightly higher Dco for eggs with holes in the air space shell. Presumably the Dco measurement during this period provides the best estimate of the actual $Do_2$ of intact eggs; the slightly higher Dco observed in eggs with holes may be a result of removing part of the barrier to carbon monoxide diffusion into the egg. Therefore, it appears that removal of the air space shell does not importantly influence the determination of Dco.

A third condition which may have artifactually influenced the measurements of Dco and oxygen consumption is the progressive fall in oxygen concentration which occured in the chamber gases during measurements of oxygen consumption. The oxygen concentration in the chamber gases, by calculation, probably reached a nadir of about 19 percent at the end of the five minute period with 18 day embryos. This two percent deficit was promptly restored at the end of the five minute measurement of oxygen consumption and the eggs were allowed to equilibrate with this normal atmosphere for about one minute before Dco was measured.

B. Interpretation of the Results

When our measurements on eggs incubated in 60 percent oxygen are compared with those obtained using eggs of the same age incubated

in room air, the high oxygen eggs show: An increased embryo weight, an increased rate of embryonic development, a decreased rate of oxygen consumption per gram of embryo and a decreased Dco per egg. Can these changes be explained? One attractive explanation is that 60 percent $O_2$ chicks are more mature at any given age than room air chicks and have had less stimulation for chorioallantoic vascular development due to the higher ambient $pO_2$.

The graph of weight against age (figure 3) shows that 60 percent $O_2$ chicks are equivalent in weight to room air chicks about 12 to 18 hours older. The increased weight in 60 percent $O_2$ agrees with the findings of Flemister and Cunningham (119) who incubated chicks at 40 pounds of air (equivalent to about 55 percent $O_2$). However, these investigators found a 40 percent increase in weight at ten days whereas, in this experiment, ten day old chick embryos incubated in 60 percent oxygen weighed about 11 percent more than the room air chicks of the same age.

A very similar relationship is apparent in the graph of stage against time of incubation (figure 4). Here is an indication that 60 percent $O_2$ chicks, at any given age, are also about 12 hours more morphologically mature.

The lower rate of oxygen consumption at any given age found in 60 percent $O_2$ chicks is also consistent with their greater weight and morphological development. Because the amount of oxygen con-

sumed per hour per gram of chick embryos decreased with increasing age, more mature embryos would be expected to consume less $O_2$/gm./hr.

A second interpretation of the lower oxygen consumption of 60 percent oxygen embryos is that the elevated $pO_2$ had an inhibitory effect (lasting at least three to five hours after exposure to room air) upon the cellular respiration of the chick embryo. The only evidence on chick embryos bearing on this suggestion comes from Needham (133c) who describes two experiments of Hasselbalch designed to study the respiratory changes of the hen's egg in increased oxygen. In 82 percent oxygen, the rate of oxygen uptake was only a quarter the normal; whereas in 79 percent oxygen, oxygen uptake occured at three times the normal rate. Oxygen consumption of men is not detectably altered by oxygen at one atmosphere (18) and the oxygen consumption of intact mice appears to be independent of increased environmental $pO_2$ up to a few minutes before death at 8 atmospheres of oxygen (138).

A third possibility is the opposite, i.e. oxygen consumption is lower in chick embryos incubated in 60 percent oxygen and exposed to room air during measurement of oxygen consumption, because blood (and therefore tissue) oxygen tensions fall too low to support a normal respiratory rate. This hypothesis presumes that the $pO_2$ within some mitrochondria is about one mm. Hg. or less (139). Such an effect occurs, for example in resting human skeletal muscle at a venous $pO_2$

of about 30 mm. Hg. (140).

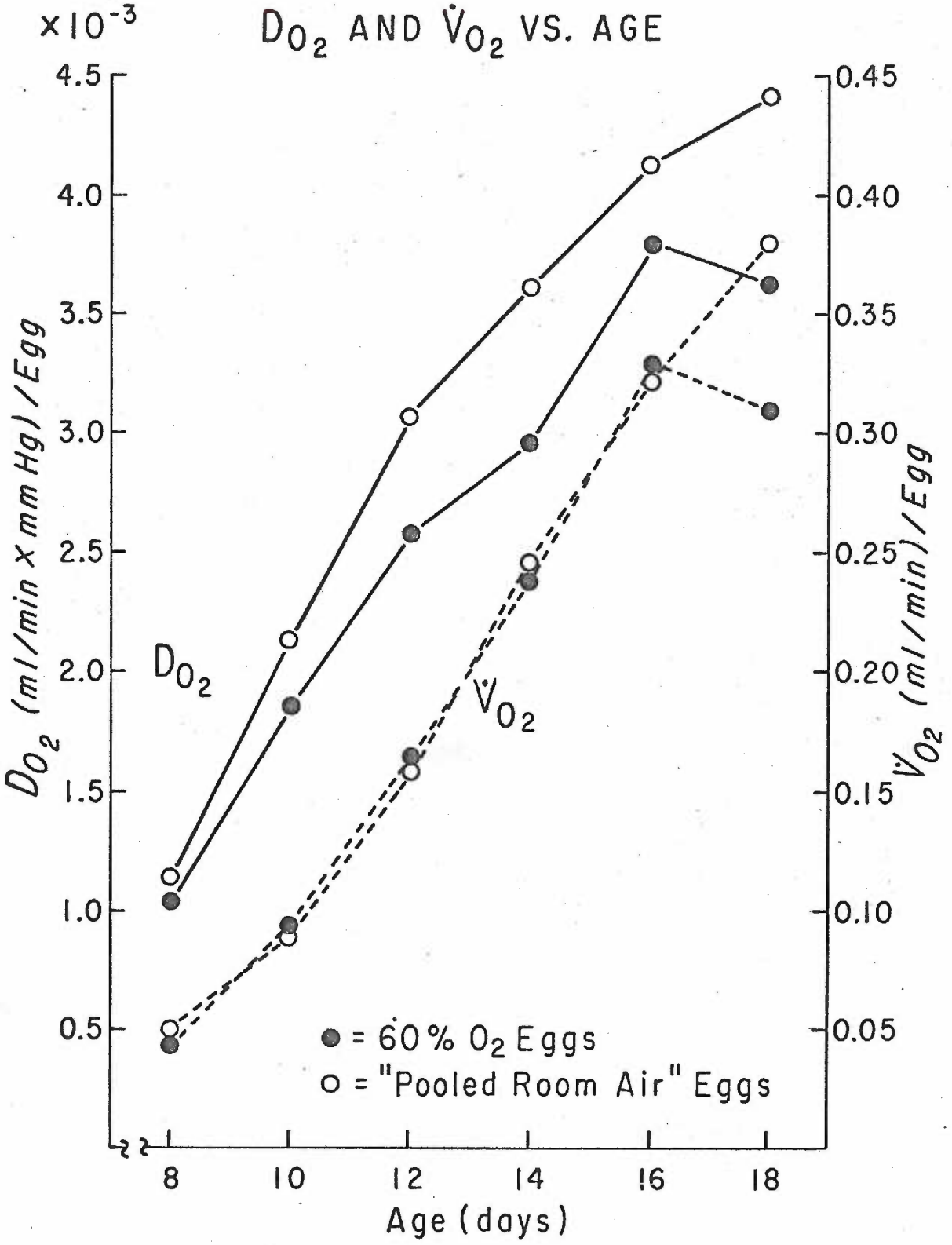
Figure 11 compares the changes of the oxygen diffusion capacity per egg with the changes of rate of oxygen consumption per egg at different ages. In these calculations, $Do_2 = 1.23$ Dco. (This relationship assumes that the relative rates of diffusion of these gases in tissures, membranes and shell is directly proportional to their relative solubilities in water at $37^{\circ}$ C. and inversely proportional to the square roots of their molecular weights (136c)). Sixty percent oxygen eggs have a lower rate of oxygen consumption per gram but a higher weight than room air eggs with the net effect that the rate of oxygen consumption per egg is about equal in both groups at all ages except 18 days.

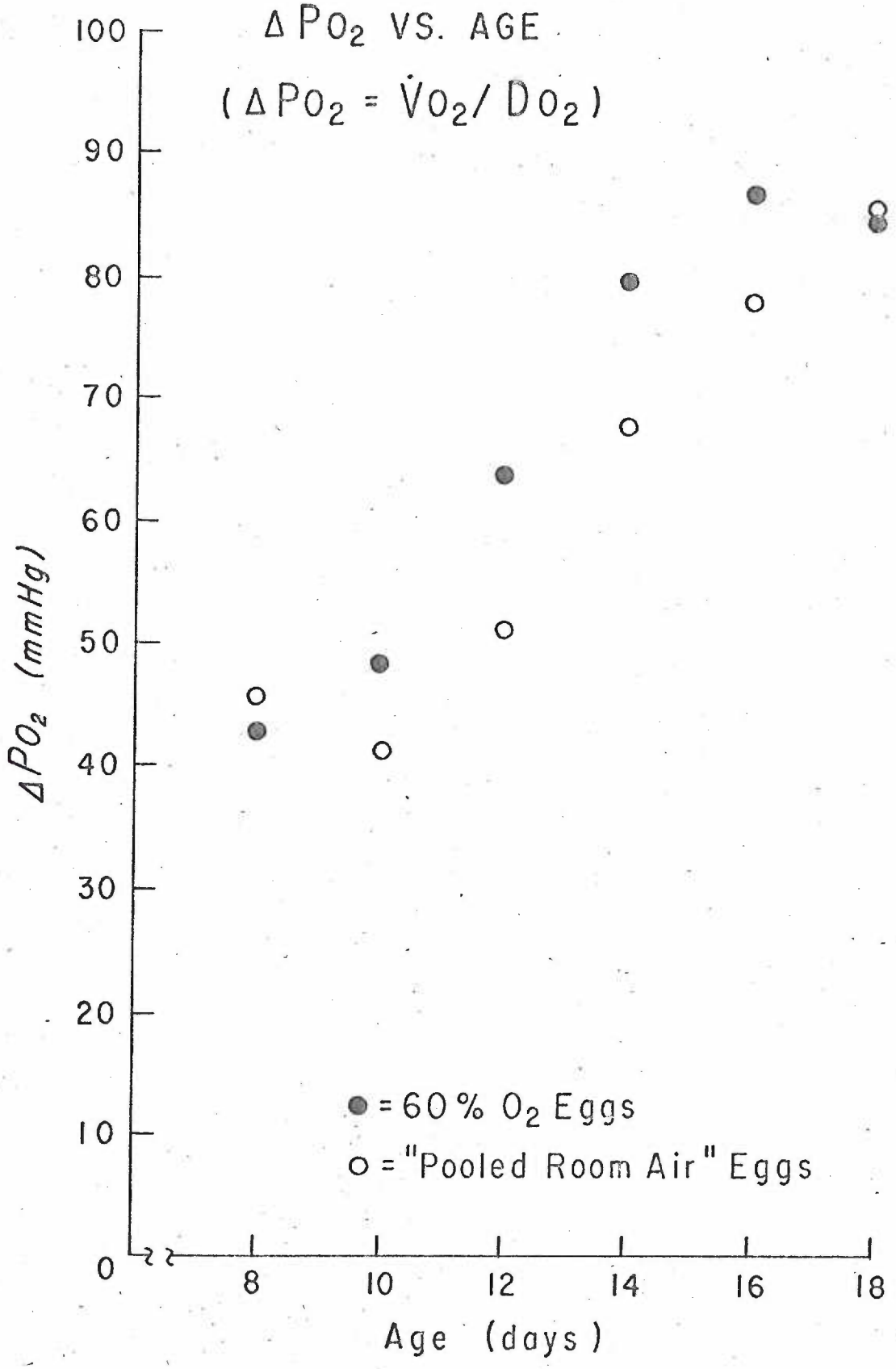
Using average values from figure 11, the rate of oxygen consumption per egg was divided by the diffusion capacity for oxygen per egg. This gives the mean $pO_2$ gradient ($\Delta pO_2$) between the outside surface of the shell and the chorioallantoic capillary blood, and this is plotted against age in figure 12. Although the curves for the rate of oxygen consumption per egg and $Do_2$ per egg (figure 11) rise approximately in parallel, the reason for the progressive increase in $\Delta pO_2$ with age is that the numerical values of oxygen consumption per egg are smaller than those of $Do_2$ at any age; therefore, a similar increase per unit time is always a proportionately greater rise in oxygen consumption than in $Do_2$.

In order to estimate the mean chorioallantoic capillary $pO_2$ dur-

Figure 11

Graph of $Do_2$ per egg and rate of oxygen consumption per egg vs. age. $Do_2 = 1.23$ Dco.


$D_{O_2}$ AND $\dot{V}_{O_2}$ VS. AGE
x10⁻³
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
$D_{O_2}$ (ml/min x mm Hg) / Egg
$\dot{V}_{O_2}$ (ml/min) / Egg
$D_{O_2}$
$\dot{V}_{O_2}$
● = 60% $O_2$ Eggs
○ = "Pooled Room Air" Eggs
8
10
12
14
16
18
Age (days)


ΔPO2 VS. AGE
(ΔPO2 = V̇O2/ DO2)
100
90
80
70
60
50
40
30
20
10
0
ΔPO2 (mmHg)
● = 60 % O2 Eggs
○ = "Pooled Room Air" Eggs
8
10
12
14
16
18
Age (days)

ing the five minute period of measurement of oxygen consumption, it is reasonable to take partial pressures of $pH_2O = 47$ mm. Hg., $pO_2 = 150$ mm. Hg. and $pN_2 = 563$ mm. Hg., assuming that essentially all $CO_2$ produced by the embryos was absorbed by the sodium hydroxide. Thus, with an external $pO_2$ of 150 mm. Hg., the 8 day chick embryo probably had a mean chorioallantoic capillary $pO_2$ of about 105 mm. Hg. This $pO_2$ steadily declined with age and at 18 days was probably about 65 mm. Hg. at the start and about 50 mm. Hg. at the end of the five minute period. Without other information it is not possible to determine the actual end-capillary $pO_2$ in the chorioallantois, but from this calculation of the mean capillary $pO_2$ and considering the shape of the blood oxygen dissociation curve it appears likely that somewhat less than 100 percent of the hemoglobin was saturated in transit through these capillaries. However, from these data one cannot state the likelihood that some 60 percent oxygen chick embryo tissues were at oxygen tensions low enough to decrease tissue oxygen consumption. The 60 percent oxygen embryos had a higher calculated $\Delta pO_2$ at all ages except 8 and 18 days (figure 12) and therefore, at 10, 12, 14 and 16 days of incubation had a lower mean $pO_2$ in chorioallantoic blood while in room air. (However, while in 60 percent oxygen, these chick embryos probably had a mean chorioallantoic capillary $pO_2$ in excess of 300 mm. Hg.) The hypothesis that 60 percent oxygen chick embryos had a lower rate of oxygen consumption per gram because

their tissues were exposed to a lower $pO_2$ which was insufficient to maintain normal respiration could be tested by conducting $O_2$ consumption measurements with 60 percent oxygen chicks in 60 percent oxygen rather than room air. In any case, it is apparent that the mean chorioallantoic capillary $pO_2$ progressively decreases with increasing incubation; the developing chick embryo may, as Sir Joseph Barcroft suggested for the lamb fetus (141), be eventually confronted with choosing between hypoxia or escape and chooses the latter on the twentieth or twenty-first day of development.

The lower Dco (and $DO_2$) of eggs incubated in 60 percent oxygen remains to be explained. An examination of the parameters influencing Dco may be useful (See appendix III). $Dco = DA/\Delta X$ where D is the diffusion coefficient for a given gas in a given medium at a given temperature; A is the surface area of the diffusion barrier, and $\Delta X$ is the thickness of the diffusion barrier. D is usually considered a constant for a given gas, but it may vary with both the concentration of the diffusing substance (142) and the structure of the diffusion medium. As an example of a structural influence upon D, fatty acid monolayers have a much lower diffusion coefficient for carbon dioxide than does the same material in bulk solution, by a factor as large as 1/10,000 (143). Theoretically, then, either D or A or $\Delta X$ might be altered as a result of, or in response to, an elevated environmental $pO_2$. However, electron micrographs have shown that the tissue layers

separating chorioallantoic blood from the inner shell membrane are already very attenuated after 14-18 days incubation in room air (130, 131) and, there is little evidence upon which to postulate changes in membrane composition or structure. Thus, the most adaptable component to changes in respiratory conditions seems to be the surface area of the capillaries. Capillaries could alter their surface area by changing their length, diameter or their density. Changes in capillary surface area could occur transiently; for example, in response to a sudden decrease in ambient $pO_2$ following incubation at a $pO_2$, the chorioallantoic capillaries might constrict. Although this would seem, teleologically, to be an inappropriate response to the need for oxygen, there is no direct evidence available to rule out such a possibility. A more permanent restriction of capillary surface area could occur as a result of a decreased rate of capillary growth. The hypothesis that differences in Dco between room air and 60 percent oxygen chick embryos are due largely to differences in capillary surface area is consistent with the observations that chick embryos exposed to 40 lbs. pressure of air for the first ten days of incubation show visible decreases in chorioallantoic vasculature (119) and when grown in 50 to 75 percent oxygen show a wider meshed capillary net with wider capillary lumens (127). All these changes support the concept of a decreased chorioallantoic capillary surface area with incubation at elevated oxygen tensions. Other less direct evidence sup-

porting this hypothesis that exposure to high oxygen tensions inhibits capillary growth comes from the observed decrease in vascularity found both in the yolk sac and intraembryonically in the chick (78), and the marked inhibition of vascular development which occurs in the immature retinas of various species (96) when the animals are exposed to elevated environmental oxygen tensions.

The formula, $Do_2 = DA/\Delta X$, can be arranged to $A=(Do_2 \cdot \Delta X)/D$. thus, if we know the value for D for the overall diffusion barrier between the capillary blood and external surface of the shell and the mean diffusion distance we could compute the surface area for diffusion. However, no values for D for the egg shell and shell membranes appear to be available. Romanoff (132b) gives values for the bulk flow of air and oxygen through the egg shell and shell membranes which are about one thousand times greater than the diffusion capacity for oxygen obtained for the whole egg in this experiment. However, bulk flow measurements on biological and synthetic membranes with pore radii of 0.5 mu or greater are considerably higher than diffusional measurements on the same material(144). Therefore, we are unable to calculate even a reasonable approximation of chorioallantoic capillary surface area.

Although the effective surface area for diffusion of the chorioallantoic vasculature is not known, it is interesting to note that the $Do_2$ of intact 18 day old eggs per egg shell surface area ($4.0 \times 10^{-3}$ ml./min.

x mm. Hg. /77 $cm^2$. = 5.2 x $10^{-5}$ ml. /min. x mm. Hg. x $cm^2$.) approximates the $Do_2$ per unit surface area of the human lung (30 ml. /min. x mm. Hg. /75 $m^2$. = 4 x $10^{-5}$ ml. /min. x mm. Hg. x $cm^2$.). This physiologic similarity may be analogous to the anatomic similarities between the chick chorioallantois and the human lung noted by Fülleborn (129)

## VI. Summary and Conclusions

Fertile hen's eggs were incubated in room air and 60 percent oxygen and examined at 8, 10, 12, 14, 16 and 18 days of incubation. Eggs incubated in 60 percent oxygen had lower diffusion capacities for carbon monoxide, lower rates of oxygen consumptions per gram of embryo, heavier embryos and more advanced embryonic morphological development than eggs incubated for the same time in room air.

The lower rates of oxygen consumption per gram of embryos incubated in 60 percent oxygen were consistent with an increased rate of growth; the lower diffusion capacities of eggs incubated in 60 percent oxygen were interpreted as probably due to less chorioallantoic capillary surface area.

Appendix I - Protocol

A. Pre-experimental

1. If the eggs were being incubated in 60 percent oxygen, the oxygen inflow to the incubator was replaced by room air.

2. An appropriate number of eggs for one experiment were candled and a hole was made in the shell over the air space in each presumably viable egg. The eggs were then recandled to confirm embryonic viability.

3. The eggs were replaced in the incubator and allowed to warm for two hours. During this time all instruments were turned on and allowed to stabilize, the carbon dioxide absorber was renewed and the complete circulating system was checked for leaks.

B. Experimental

At the end of the two hour warming period the experiment was begun. Each experiment consisted of measurement of oxygen consumption, diffusion capacity for carbon monoxide, the developmental stage and weight of each embryo (and its blood hemoglobin concentration in the second room air group).

1. Before starting, the egg chamber was submerged in the water bath for at least five minutes. The setting of the water bath temperature regulator was then increased by 0.8° C. to 40.3° C., to speed the initial warming of the chamber gases during the first minute that the chamber was resubmerged.

The chamber was lifted from the water bath and the chamber door opened. Three holed eggs were removed from the incubator and quickly candled to assure continued viability. The eggs were promptly placed in the egg chamber, the chamber door was sealed and the entire chamber resubmerged in the water bath. (If, during the approximately one minute that the chamber was out of the water bath the temperature of the chamber gases fell below 33° C., the eggs were replaced in the incubator and the chamber was rewarmed.) The stopwatch was started.

After 30 seconds the setting of the water bath temperature regulator was decreased to 39.5° C. Gaseous expansion due to warming was nearly complete (except for about 0.9 cc.) at the end of one minute if the bath temperature was increased 0.8° C. for the first 1/2 minute. During the first one minute of warming the circulating chamber gases were permitted to escape into the external atmosphere.

At the end of one minute, all openings to the chamber, except that to the manometer, were sealed and the temperature of the chamber gases recorded (38.7 to 39.3° C.)

2. Oxygen was consumed during the subsequent five minutes and at the end of this time the resulting pressure change was recorded and the temperature of the chamber gases was recorded (39.3 to 39.7° C.). If the temperature of the chamber gases was outside these limits the measurement of oxygen consumption was considered unreliable.

3. The oxygen reservoir was connected to the chamber and

the consumed oxygen was replenished and the remaining chamber openings were all sealed.

For five seconds 100 percent carbon monoxide was allowed to flush a 0.25 ml. syringe from the needle end outward through a hole in the syringe barrel at the plunger end. The plunger was then depressed to a constant distance against a standard plunger stop and the CO was promptly injected into the circulating system. The stopwatch was restarted at the instant of injection. The removal of CO from the external gas by the eggs was recorded for at least five minutes.

4. The eggs were removed from the chamber. In the second room air group, blood for measurement of hemoglobin concentration was obtained from one of the three eggs. The small hole used for vessel puncture was patched with masking tape.

5. The volume of each of the three eggs was measured by saline displacement.

6. The chick embryos were removed from the shell, gently washed, and with the use of a dissecting microscope or binocular loupes, staged as indicated in Appendix II.

7. Each of the chick embryos was weighed.

Appendix II - Morphological Staging

(According to Hamburger and Hamilton, in Lillie's Development of the Chick, Holt, Rinehart and Winston, New York, 1952, pp. 87-91) (98c).

| Stage | Criteria |
|---|---|
| 32 | Webs between toes concave<br>Tip of mandible to tip of beak<br>6-8 scleral papillae |
| 33 | 3 rows of feather germs on tail<br>13 scleral papillae |
| 34<br>(about 8 days) | Webs concaved and arched<br>Nictitating membrane 1/2 to scleral papillae<br>14 scleral papillae |
| 35 | Webs gone<br>4 rows feather germs on inner eye margin<br>Nictitating membrane 3/4 to scleral papillae |
| 36<br>(about 10 days) | Comb with slight serrations<br>Slight labial groove on mandible, prominent labial groove on maxilla<br>Nictitating membrane between scleral papillae and cornea. |
| 37 | Prominent comb serrations<br>Nictitating membrane to cornea; upper lid to cornea.<br>Beak =3. 0 mm. |
| 38<br>(about 12 days) | 2 rows of feather germs on lower lid<br>Lower lid over 2/3 of cornea<br>Beak = 3. 1 mm. |
| 39 | 4 rows of feather germs on lower lid<br>Prominent overlapping of scales on legs<br>Beak = 3. 5 mm. |
| 40<br>(about 14 days) | Beak = 4. 0 mm. |

| Stage | Criteria |
|---|---|
| 41 | Beak= 4. 5 mm. |
| 42 (about 16 days) | Beak= 4. 8 mm. |
| 43 | Beak= 5. 0 mm. |
| 44 (about 18 days) | Beak= 5. 7 mm. |

Appendix III - Analysis of Dco

A. Theoretical

If an egg is placed within a closed chamber and a gas in injected into the chamber, the gas will diffuse into the egg and the rate of fall of the partial pressure of the gas outside the egg will be $dp/dt = -k (p_{out} - p_{in})$ where p represents partial pressure and k is a constant. If the gas diffusing into the egg is absorbed so that its partial pressure within the egg does not rise as diffusion occurs, then the pressure gradient remains equal to the partial pressure of gas outside the egg and $dp/dt = -k \cdot p_{out}$. Carbon monoxide is very tightly bound to hemoglobin and in the presence of adequate quantities of hemoglobin, the partial pressure of CO (pCO) in blood rises very little as CO concentration rises. Therefore CO approximates the above ideal situation. Rearranging the last equation we have $dp/p_{out} = -kdt$ which integrates (between $t = t_1$ and $t = t_2$) to $\ln p_2 - \ln p_1 = -k(t_2 - t_1)$. If $t_1$ = time zero, $\ln p_2 = -kt_2 + \ln p_1$ which has the form of $y = ax + b$ of a straight line. Thus if we plot $\ln p_2$ on the ordinate and t on the abscissa, the slope will be (-k) and the ordinate intercept will be $\ln p_1$, or the natural log of the original partial pressure outside the egg. The last equation can be transformed into the familiar exponential form of $p_2 = p_1 e^{-kt_2}$. Either of the last two equations will define the partial pressure outside the egg ($p_2$) at any time ($t_2$), assuming that no significant increase in partial pressure of the test gas inside the egg occurs.

Fick's law of diffusion is $dQ/dt = -DA(dc/dx)$, where $dQ/dt$ is the rate of flow (ml./sec.) of the diffusing substance along a concentration gradient $(dc/dx)$. $(dc/dx)$ refers to concentration changes over infinitessimally short distances but becomes $(\Delta c/\Delta x)$ when the individual concentration gradients of the whole diffusion barrier are taken as one. (Although this is strictly true only in the steady state, a steady state condition is probably closely approximated during the period of measurement of Dco in the present experiments (142).) A is the surface area for diffusion ($cm^2$.), D is the diffusion coeficient ($cm^2$./sec.) which is considered a constant and $\Delta x$ is the thickness of the diffusion barrier (cm.). Rearranging Fick's equation, $\frac{dQ/dt}{\Delta c} = \frac{-DA}{\Delta x}$; converting $\Delta c$ to $\Delta p$, where $\alpha$ is the solubility of the diffusing gas in the diffusion barrier in ml. gas per ml. tissue per mm. Hg., and assuming that carbon monoxide is the diffusing gas, we obtain the so-called diffusion capacity for carbon monoxide (Dco): $\frac{dQ/dt}{\Delta pCO} = -\frac{\alpha 60DA}{\Delta x}$, where 60=sec./min. The left hand side of the equation indicates the units of Dco to be ml. gas diffusing per minute per mm. Hg. partial pressure difference of CO, and the right hand side of the equation reveals that the Dco is directly proportional to the solubility of the gas, the diffusion coefficient of the gas and the surface area for diffusion and inversely proportional to the thickness of the diffusion barrier. Substituting Dco for $\frac{\alpha 60DA}{\Delta x}$, $dQ/dt = -Dco\Delta pCO$. When pCO inside = 0, $dQ/dt = -Dco \cdot pCO_{out}$. Dividing by the volume of the chamber exter-

nal to the eggs ($V_o$) and multiplying by $\gamma = (P_{barometric} - P_{H_2O})$, we obtain $dp^o/dt = -\left(\frac{Dco \cdot \gamma}{V_o}\right) \cdot p^o$, which is identical in form to the equation originally considered on page 101, $dp/dt = -kp^o$.

Integrating as before yields,

$$\ln p_2^{\,o} - \ln p_1^{\,o} = -\left(\frac{Dco \cdot \gamma}{V_o}\right) \cdot t_2, \text{ where } t_1 = 0.$$

Converting to base 10 logs gives,

$$\log p_2^{\,o} - \log p_1^{\,o} = -\left(\frac{Dco \cdot \gamma}{2.3V_o}\right) \cdot t_2$$

and converting to percentage of $C_1$,

$$\log\left(\frac{p_2^{\,o}}{p_1^{\,o}} \times 10^2\right) = -\left(\frac{Dco \cdot \gamma}{2.3V_o}\right) \cdot t_2 + \log 10^2$$

which becomes

$$\log \%\, p_1^{\,o} = -\left(\frac{Dco \cdot \gamma}{2.3V_o}\right) \cdot t_2 + 2$$

This equation describes a straight line of slope $-(Dco \cdot \gamma / 2.3V_o)$ and intercept on the abscissa ($\log \%\, p_1^{\,o}$) at 2. The slope can be defined as $\frac{\Delta \log \%\, p_1^{\,o}}{t_2} = -\left(\frac{Dco \cdot \gamma}{2.3V_o}\right)$ and rearranging,

$Dco = -\left(\frac{\Delta \log \%\, p_1^{\,o}}{t_2}\right) \cdot \left(\frac{2.3V_o}{\gamma}\right)$. So, if the slope of the CO decay curve is known, the Dco may be calculated. Extrapolation of the curve to where the original concentration falls to one-half gives a good estimate of the slope. Call $t_{50}$ the time at which the partial pressure is 50% of the original value. Then, $Dco = (\log 100\% - \log 50\%) \cdot \left(\frac{2.3V_o}{t_{50} \cdot \gamma}\right)$

and, $Dco = (0.301)(2.302)\left(\frac{V_o}{t_{50} \cdot \gamma}\right)$; let $\gamma$ = 700 mm. Hg. ,

then, $Dco = \frac{0.693}{700} \cdot \frac{V_o}{t_{50}} = \frac{V_o}{(1400)(0.722)\, t_{50}}$

thus $Dco = \frac{V_o}{(1010)\, t_{50}}$.

The same value for Dco may be obtained from the definition of Dco by finding the mean time integral of the equation $p_2 = p_1 \cdot e^{-kt}$ and substituting the value of k from the relationship $\ln p_2 = \ln p_1 - kt$ with values of $p_2$ and $p_1$ taken as 0.5 and 1.0 respectively, at $t = t_{50}$.

B. Methods of Calculation*

In order to determine the slope of the decay curve the percent $p_1$ values may be taken from the linear recording of the CO partial pressure and plotted on semilog paper against time. A straight line is drawn through the best fit of these points. The following method of determining the $t_{50}$ does not require replotting these values on semilog paper and gives a consistent interpretation of the best fit for a straight line through such plotted points.

Consider the equation of a straight line, $y = ax + b$, which in our case is $\log \%\, p_1 = at + b$. It is desired to find a and b and these can be determined by solving two simultaneous equations containing a and b, with one equation describing the first half of the 3 1/2 minute time interval and the second equation describing the second half. Choosing to use percent partial pressures at every half minute, these equations are

then, $\sum(\log \% p_1) = a \sum t + \sum b$ (for 0 to 1 1/2 min.)

$\sum'(\log \% p_1) = a \sum' t + \sum' b$ (for 2 to 3 1/2 min.)

where a is the common slope and b the common intercept.

Because $\sum t$ = 3 minutes and $\sum' t$ = 11 min., and because b is taken four times for each equation,

$$\sum (\log \% p_1) = 3a + 4b$$

$$\sum'(\log \% p_1) = 11a + 4b.$$

Solving as simultaneous equations,

$$a = \frac{\sum'(\log \% p_1) - \sum(\log \% p_1)}{8}$$

$$b = \frac{11\sum(\log \% p_1) - 3\sum'(\log \% p_1)}{32}$$

Since, $\log \% p_1 = at + b$ then $\log (50) = a(t_{50}) + b$, so that

$$t_{50} = \left(\frac{\log (50) - b}{a}\right) = \left(\frac{1.699 - b}{a}\right)$$

By listing the first four % $p_1$ values in one column and the second four in another column and placing the respective logs beside each value of % $p_1$, a and b and hence $t_{50}$, may be rapidly found.

In the experiments described, about 15 seconds were required for complete mixing of the carbon monoxide and probably about 20 to 30 seconds were required to closely approximate the steady state (142), therefore, the first point (or $p_1$) was taken as the partial pressure at 30 seconds and the remaining points were considered as percentages of this partial pressure.

*This method is an adaptation of the method of averages described in Daniels, F., Mathematical Preparation for Physical Chemistry, McGraw-Hill, 1956 pp. 235-237.

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