EMBRYOGENESIS IN 100% O_2 AT REDUCED PRESSURE*

HAROLD S. WEISS and MICHEL GRIMARD**

Environmental Physiology Laboratory, Dept. of Physiology, College of Medicine, The Ohio State University, Columbus, Ohio, U.S.A.

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Abstract. Fertile chicken eggs were incubated in an altitude chamber in a near 100% O_2 atmosphere at 225 torr. Both N_2 and CO_2 were kept under 0.5%. Temperature was a standard 37.5° but a high relative humidity of 90% was required to prevent dehydration. In ten trials involving 382 eggs, hatchability averaged 21% of controls and weight of chicks was 11% less than controls, but embryo mortality was distributed similarly. Low pressure *per se* and small differences in O_2 tension may have affected the results, but similarities to incubation in 21% O_2–79% He call attention to absence of nitrogen as a possible explanation.

1. Introduction

Embryo development has served as a sensitive test in the search for artificial atmospheres suitable for life support in sealed systems. In connection with the need for inert gas in such atmospheres, incubation studies with fertile chicken eggs have suggested that He may not adequately replace N_2 inasmuch as embryonic mortality was doubled in 21% O_2–79% He compared to air (Weiss *et al.*, 1965). The increased mortality could not be corrected by environmental manipulations involving temperature, humidity or insulation which were designed to compensate for the higher heat conductivity of He compared to N_2 (Weiss and Wright, 1968). One of the questions raised by these results was whether the depression in hatchability was related to the presence of He or the absence of N_2. In the present study, the question was examined by incubating fertile eggs in an atmosphere essentially devoid of all inert gas, accomplished by using 100% O_2 at reduced pressure. Prior studies of this type seem to have been limited to 3–4 days of embryonic development (Cook, 1945; Allen, 1963).

2. Procedure

The low pressure incubation system consisted of a cylindrical metal altitude chamber approximately 2 m long and 1 m diam. Sliding in and out of the metal cylinder on a set of tracks was a sealed polyvinyl chamber (modified after gnotobitic isolators) in which the eggs were held. Before insertion into the altitude chamber, the isolator was loaded with fertile eggs, a supply of water for maintaining humidity and a tray of soda lime for adsorption of CO_2. It was then flushed with 100% O_2 until less than 1% N_2 remained. Thereafter the isolator was supplied with 100% O_2 from a pressurized cylinder thru a demand-type oxygen mask regulator located within the altitude chamber. The regulator kept the flexible plastic isolator inflated with a pressure 1–2

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** Dr Grimard's present address: Schering Corp., 3535 Transcanada Highway, Pointe Claire 730, Quebec, Canada.

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cm H_2O above that in the altitude chamber per se. Any leaks in the isolator were therefore outboard, insuring a near 100% O_2 atmosphere (Grimard, 1970).

A pressure of 225 torr was selected as a P_{O_2} likely to avoid problems from O_2 toxicity and still provide a margin of safety against hypoxia (see discussion). Pressure was maintained in the altitude chamber by means of a sensor (I^2R Therm-o-Watch) mounted on a Hg manometer. Standard incubation temperature of 37.5° was used (thermistor controlled, YSI) but initial studies showed that a relative humidity around 90% (by hair hygrometer calibrated in room air) was required to prevent excessive dehydration of eggs (Taylor, 1949). The high moisture levels were obtained by water pans placed in the bottom of the isolator. A small, continuously operating fan within the isolator aided in gas mixing and heat distribution.

Fungal and bacterial growth appeared to be promoted in and on the eggs by the isolator conditions and caused at least 5 runs to be discarded. The problem was reduced to a manageable level by: (1) using unwashed eggs; (2) preventing moisture which condensed on the isolator walls from dripping onto the eggs; (3) dusting the eggs (including controls) with nystatin (Mycostatin), and anti-fungal agent; and (4) spraying the isolator before use with a peracetic acid solution.

Fertile eggs in the 40–60 g range were obtained from the University Poultry Dept's White Leghorn flock, weighed, and set pointed end down. They were turned thru 90° once a day for the first 18 days by a remote operating linear actuator (Grimard, 1970) thus avoiding the need for daily decompression and recompression of the altitude chamber. On the 18th day, the chamber was returned to ground level, the isolator wheeled out, and the eggs laid horizontal. A test of gas composition was made at this time, using a Beckman E-2 for O_2, Beckman LB-1 for CO_2 and a Barber-Coleman gas chromatograph for N_2. All manipulations were carried out by means of rubber gloves incorporated in the isolator wall and a flushable gas lock, insuring minimal disturbance of the 100% O_2 atmosphere. The system was returned to altitude until the 22nd day, when the trial terminated.

A typical trial involved 18–30 eggs each in the isolator and in the control system, which was a small, commercial, forced-draft incubator (American-Lincoln) located on an adjacent lab bench. Unhatched eggs were weighed after incubation to evaluate weight loss. All unhatched eggs were opened and if fertile, time of death of embryo estimated as during the first, second or third week of incubation. Chicks which hatched were weighed and some were raised for short periods to observe performance. Hatchability was determined as the percent of fertile eggs which produced live chicks, and relative hatchability was the ratio of the hatchability in the isolator system to control hatchability. Mortality distribution was calculated as a percent of the number of unhatched fertile eggs. Contingency Chi Square, 't' tests between means and correlation analyses were used to evaluate results (Snedecor, 1956).

A small series of additional 'control' runs was carried out in the isolator-altitude chamber incubation system. The system was loaded and sealed but not taken to altitude and the atmosphere remained room air. Relative humidity was held between 45

and 65 %. The isolator was kept inflated from a cylinder of compressed air, although in the last days of incubation, enrichment with O_2 was required to replace O_2 utilized by the embryos.

3. Results

Of the 23 separate incubation starts with 100 % oxygen at 225 torr, 13 were aborted or discarded because of technical problems, i.e., failure in pressure or temperature control, excessive gas leakage, bacterial and fungal contamination, etc. The 10 remaining acceptable experiments involved a total of 382 fertile eggs. Table I indicates that hatchability in air averaged 66 % (range 31 to 100 %) and was 4–5 fold greater than in 225 torr of 100 % oxygen (relative hatchability in O_2 of 0.21). Mortality distribution, however, was remarkably alike for the control and altitude embryos (Table II). For the three trials in which air at ground level pressure was used in the altitude system,

TABLE I

Hatchability of fertile chicken eggs in 100 % oxygen at reduced pressure vs. air at ground level (10 trials)

Atmosphere	Hatched		Not Hatched		
	No.	% of total	No.	% of total	No. total
Oxygen at 225 torr	33	14.2[a,b]	200	85.8[a]	233
Air at 747 torr	99	66.4[c]	50	33.6	149

[a] Significant at the 1 % level by Chi Sq. analysis (2 × 2 contingency test).
[b] Relative hatchability (O_2/air) = 0.21.
[c] Correlation (r) between hatchability in air and relative hatchability = − 0.73.

TABLE II

Mortality distribution of embryos incubated in 100 % O_2 at reduced pressure vs. air at ground level (10 trials)

Atmosphere	Time of Death						
	First Week		Second Week		Third Week		
	No.	% of total	No.	% of total	No.	% of total	Total
Oxygen at 225 torr	36	18.0[a]	25	12.5[a]	139	69.5[a]	200
Air at 747 torr	9	18.0	5	10.0	36	72.0	50

[a] Not significant by Chi Sq. analysis (2 × 3 contingency test).

relative hatchability in the isolator was 0.90. The correlation between hatchability in air and relative hatchability in 100 % O_2 for the 10 altitude trials was negative, $r = -0.73$.

As observed from daily recordings, temperature was essentially the same in the isolator and control systems, averaging between 37.3 and 37.7°. Relative humidity ranged between 85 to 95 in O_2 and 45 and 65 in air. Pressure in the O_2 system averaged 225 torr and 747 torr in air. N_2 content in the isolator was always below 0.5 % (ie, P_{N_2} around 1 torr or less) and CO_2 did not rise above 0.5 % (gases were measured at start, after 18 days and at end of incubation, and also whenever else the system had to be returned to ground level).

Egg weight loss tended to be higher in O_2 (Table III), but only averaged 14.5 % of initial weight and was not statistically different from the loss in air. The correlation between the air-O_2 difference in weight loss and the air-O_2 difference in hatchability yielded a small and non significant r of -0.33. Random samples of chick weights

TABLE III

Egg weight loss after 22 days' incubation in 100 % O_2 at reduced pressure vs. air at ground level

Atmosphere	No trials	Mean ± SE	
		Grams	Percent
Oxygen at 225 torr	10	8.4 ± 1.2[a]	14.5 ± 2.0[a]
Air at 747 torr	10	6.8 ± 0.6[b]	11.5 ± 0.9

[a] Not significant by 't' test.
[b] Correlation between air-O_2 difference in egg weight loss and air-O_2 difference in hatchability = − 0.33.

TABLE IV

Hatching weight of chicks incubated in 100 % O_2 at reduced pressure vs. air at ground level

Atmosphere	No.	Mean ± SE Grams
Oxygen at 225 torr	21 chicks, 7 trials	34.8 ± 0.8[a]
Air at 747 torr	40 chicks, 6 trials	38.2 ± 0.8[b]

[a] Significant at 1 % level by 't' test.
[b] Correlation between air-O_2 difference in egg weight loss and air-O_2 difference in chick weight = − 0.14.

indicate that those in O_2 were significantly smaller (by 11 %) than those in air (Table IV). A correlation between air-O_2 difference in egg weight loss and air-O_2 difference in chick weight yielded a small-non significant r of -0.14. Chicks hatched in the isolator system under ground level conditions weighed the same as those from the commercial incubator. Several groups of O_2 chicks raised for approximately two weeks showed normal growth and livability.

4. Discussion

A 100 % O_2 atmosphere at 225 torr clearly is deterimental to embryonic development, reducing hatchability of fertile eggs to 21 % of that in air and reducing size of chicks which do hatch by 11 %. Perhaps it is remarkable that any chicks hatched at all, considering the marked retardation and high rate of abnormalities observed after 3–4 days incubation under similar conditions (Cook, 1945; Allen, 1963). The depression in hatchability and chick size would not appear to be due to any intrinsic mechanical or physical feature of the isolator-altitude chamber incubation system since chicks equivalent to control in numbers and size were obtained when the ambient gas was air at ground level pressure.

Conflicting possibilities appear in trying to relate the O_2 effects on the embryos with humidity-dehydration differences between incubation systems. Thus, the 85–95 % RH level of the altitude system might be expected to depress hatchability (Robertson, 1961a), presumably by inhibiting proper evaporation from the egg. However, egg weight loss was actually greater in the altitude eggs, altho within normal limits (Robertson, 1961b). Furthermore, the correlations between air-O_2 difference in either hatchability or chick size vs the air-O_2 difference in egg weight loss were small and non-significant, casting doubt on any major effect of moisture flux on the results.

The fact that the overall control hatchability of 66 % was on the low side does not appear to have biased the results, since the correlation of -0.73 between hatchability in air and relative O_2 hatchability suggests the better hatches were the ones most adversely affected by the O_2 atmosphere. Indirect temperature effects, as was postulated in 21 % O_2–79 % He (Weiss and Wright, 1968) seems unlikely either, since heat conduction is similar in O_2 and N_2 (Radford, 1964). The normal distribution of embryo mortality over the 3 week incubation period suggests an intensification of the usual causes of embryo death, rather than the operation of specific lethal factors (Robertson, 1961a; Smith *et al.*, 1969). It may also be noted that the O_2 exposure apparently left no residual mark on the chicks which did hatch, since they survived and grew normally for at least two weeks.

Obvious similarities exist between the present 225 torr–100 % O_2 studies and the previous ground level studies with 21 % O_2–79 % He in such aspects as decreased hatchability but normal distribution of embryo mortality, smaller chick size at hatching but normal growth subsequently, and the inability to relate these results in either case to temperature, humidity or physical-mechanical features of the experimental incubation systems. The composition and pressure of the gas in contact with

the eggs thus appear to be the important factors, and in the present study differ from room air in (1) low total pressure, (2) small deviations in O_2 concentration, and (3) near absence of N_2.

The effect of low total pressure per se on embryogenesis can not be evaluated precisely, since it is usually compounded by hypoxia or absence of nitrogen. Altitude does depress hatchability, but at least up to elevations of 2200 m (580 torr), the depression can be reversed by supplemental O_2, suggesting that the altitude effect is primarily related to hypoxia (Altman and Dittmer, 1966). It is not known to what altitude the hypoxia concept can be extended, but it certainly does not seem to apply to the approximately 9000 m of the present study. Cook (1945) incubated eggs for up to 72 hours in 100 % O_2 at 190 torr and concluded that the marked retardation in development he observed was due to low total gas pressure. Allen (1963) on the other hand, concluded that the circulatory abnormalities seen in 4 day embryos incubated in 100 % O_2 at reduced pressure was due to low N_2 levels, since normal development occurred if P_{N_2} was 80 torr or more. No one appears yet to have extended Allen's (1963) experiments to the full 21 day incubation period.

Calculation of oxygen tensions during incubation in 100 % O_2 at 225 torr indicates the possibility of both slight hyperoxia and slight hypoxia. The P_{O_2} in the isolator should be around 183 torr, after deducting 42 torr for P_{H_2O} (90 % saturation at 37.5 °C). For the controls incubating in air at 747 torr and 60 % RH, the equivalent P_{O_2} would be around 151 torr [i.e., 0.21 (747–28)]. The higher P_{O_2} in the altitude system corresponds approximately to 25 % O_2 at ground level, which seems well within the tolerance limits for normal hatchability of the embryo (Taylor and Kreutziger, 1969), even the O_2 toxicity effects may be augmented in the absence of inert gas (Nelsen, 1955; Stephenson, 1969).

In contrast to slightly elevated O_2 tension in the isolator, P_{O_2} in the air space of the altitude eggs may fall below that in air during the last few days before hatching. Using the air space P_{CO_2} values of 46–63 torr before hatching (Beattie, 1964) and a saturation P_{H_2O} of 47 torr, the theoretical maximum air space P_{O_2} ranges from 132 to 115 torr for the eggs in 100 % O_2 at 225 torr vs 137–134 torr for the eggs in air at ground level. However, in view of the wide range in air space P_{O_2} compatable with normal numbers of chicks (Visschedijk, 1968) a 5–19 torr deficiency in P_{O_2} seems unlikely to have had any appreciable effect.

An explanation based on the absence of N_2 is attractive because it is a factor common to both the earlier 21 % O_2–79 % He (Weiss *et al.*, 1965; Weiss and Wright, 1968) and the present 100 % O_2 studies. One might even ascribe the higher embryo mortality in the present 100 % O_2 experiment (relative hatchability 0.21 vs 0.45 in He) to the lower residual N_2 levels achieved in the hypobaric system (P_{N_2} of 1 torr or less compared to 7–15 torr previously). One alternate possibility that should be considered is that rather than a specific requirement for gaseous nitrogen in embryogenesis, the results may indicate a need for an 'inert' gas molecule closer to N_2 in physical characteristics than is He, perhaps as suggested by satisfactory incubation studies in 21 %

O_2–79 % neon (Weiss *et al.*, 1968). However, as a perusal of the current literature indicates, there is little agreement regarding the role, if any, of these 'inert' gases at low pressure on tissue and cell function (Akers and Thompson, 1969; Grimard, 1970; Hamilton *et al.* 1970; Longmuir *et al.*, 1968; Neville and Maio, 1969; Rodgers *et al.*, 1969; Schreiner, 1968).

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