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Carbon monoxide and the embryo

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ABSTRACT Mammals are homeotherms and expend considerable energy maintaining their body temperatures. The temperature of a mammalian embryo on the other hand is maintained by the mother and the embryo can devote its metabolic energy to growth and development. The mammalian embryo is acting as a poikilotherm and its energy needs are thus considerably less than if it were a comparably sized homeotherm. The energy requirements of the preimplantation rat embryo are generated by anaerobic metabolism. As it grows, aerobic metabolism develops. In culture, the addition of carbon monoxide to the perfusing gas for early rat embryos has a much smaller effect than decreasing the oxygen concentration. Carbon monoxide appears to be a relatively mild toxicant until the embryo is much larger, is depending much more on transport of oxygen by red blood cells, and the fraction of required metabolic energy produced by anaerobic metabolism has become quite small. The effect from smoking during gestation may be either by the concomitant reduction in food intake or a more direct toxic effect from some components in the smoke. Carbon monoxide does not seem to be the culprit. The possible mitigating effect of a compensatory increase in fetal hematocrit in response to any hypoxia must also be considered. Humans have no yolk sac placenta as rodents do, but if the switch from anaerobic to aerobic metabolism is correlated with the stage of development, then carbon monoxide exposure should not represent any significant risk to the human embryo until later in gestation.

KEY WORDS: *embryos, carbon monoxide, growth, oxygen, COHb*

Introduction

All living things require energy for maintenance, development, growth, work (movement) and reproduction. How the available energy is apportioned depends on the nature of the organism and the stage in the organism's life cycle. In poikilotherms, the external environment supports the organism's temperature which reduces the demand for maintenance energy and allows a large part of the energy generated by metabolism that is not required for movement or reproduction to be used for development or growth. In homeotherms, a considerable amount of metabolic energy is devoted to maintaining the body temperature. Embryos of mammals, which post-partum are homeotherms, rely on the mother to maintain their temperature *in utero* and so have poikilothermic characteristics. Most of the metabolic energy they generate can then be spent on cell division, development and growth.

Anything that interferes with energy generation in a living organism forces it to make adjustments in what it can do. The interference may involve preventing the delivery of oxygen to the cells or inhibiting the metabolism within the cells. For the adult aerobic organism the choices are often very limited and, if the interference with energy production becomes severe enough, the result is death. For the embryo, on the other hand, the options do not appear to be so limited. Survival is sometimes possible under conditions that would be lethal for the adult.

Carbon monoxide converts hemoglobin to carboxyhemoglobin (COHb), thereby removing it as an oxygen transport vehicle. The affinity of adult hemoglobin for carbon monoxide is over 200 times greater than that for oxygen, and the affinity of fetal hemoglobin for CO is not much smaller. CO also affects energy metabolism within the cells by inhibiting some respiratory enzymes. This paper discusses the effect of carbon monoxide on embryos and the way that early embryos respond to such a challenge.

Energy requirements

Hemmingsen (1960) shows that a plot of metabolic rate (calories per hour) versus body weight fits a power function of the form

$$\text{metabolic rate} = k \cdot \text{weight}^a$$

or in its straight-line logarithmic form

$$\log[\text{metabolic rate}] = \log k + a \cdot \log[\text{weight}]$$

Hemmingsen analyzed data from a large number of aerobic unicellular, poikilothermic and homeothermic species over an enormous range of size and fitted the results to the logarithmic form of the equation. He obtained a consistent value of a = 0.751+/-0.015 for the data from all three classes of organisms. The lines for

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the three classes were displaced as represented by different values for the constant term. He found:

for unicellular organisms: log k = -4.076 +/- 0.110

for poikilotherms: log k = -3.161 +/- 0.068

for homeotherms: log k = -1.706 +/- 0.110

Figure 1 shows these curves based on the mean values for the parameters. Schmidt-Nielsen (1970) refers to these results as the "3/4 power law" and gives a general discussion of the problem of the scaling of metabolic rate with size.

Since a mammalian embryo's temperature is maintained by its maternal surroundings, it is perhaps reasonable to assume that at the stage when it is fully aerobic its metabolic requirements would be more poikilothermic than homeothermic. Thus, we can consider that embryos can use their available energy for development, growth and that part of maintenance other than thermoregulation.

Kleiber *et al.* (1943) determined the oxygen consumption of day 13 rat embryos both with and without their extraembryonic membranes. He observed that embryos without their membranes having an average dry weight of 8.0 mg consumed oxygen at an average rate of 55 mm^3 per hour. For the embryos in intact membranes, the average oxygen consumption was 67 mm^3 per hour and, when the membranes were slit open but otherwise left attached to the embryos, the average consumption was 81 mm^3 per hour. The embryo plus membranes had an average dry weight of 13.9 mg. One does not know how close these oxygen consumptions are to what would be the case *in vivo*. Without its membranes, the embryo loses the oxygen transport contribution of the yolk sac and allantoic placental circulations. At the same time, the diffusion distance from the culture medium to the embryo is considerably reduced which should enhance oxygen transfer. Kleiber's values, even if less than the oxygen utilization *in vivo*, are at least illustrative of the relative oxygen consumption rates of these tissues.

Based on the energy production of homeotherms, Kleiber *et al.* (1943) estimate that the embryo without its membranes should generate 59 calories per day from aerobic metabolism. The membranes apparently also consume considerable oxygen. If we consider the ratio of dry weights of the embryo plus membranes to the naked embryo and use the metabolic line for homeotherms, the embryo with its membranes should generate about 89 calories per day.

For an energy equivalent of 4.7 calories per milliliter of oxygen (Kleiber *et al.* 1943), the oxygen consumption of 81 mm^3 per hour (1.94 ml/d) is equivalent to an energy generation rate of 9.1 calories per day. This is a factor of 10 smaller than the prediction for homeotherms. While an embryo *in vitro* may not be in a euoxic state, it would be surprising that embryos in culture would do as well as they do if the cellular oxygen levels were only 10 % of what was required for euoxia.

If the embryo falls on the homeothermic curve, the measured oxygen consumption is inadequate to supply the apparent energy requirement of the embryo. If this is the case, then the suggestion remains, which we will later discuss further, that anaerobic metabolism may still be contributing a substantial part of the fetal energy at this age. However, the poikilothermic energy consumption curve is a factor of 29 lower than the homeothermic curve as given by the difference between the "log k" terms reported by Hemmingsen (1960).

Hemmingsen determined the poikilothermic curve with data obtained at 20°C while the homeotherm curve was based on data at 39°C. The metabolic rate of organisms increase with temperature in a rather complex way that is somewhat approximated by the Arrhenius equation for chemical reaction rates,

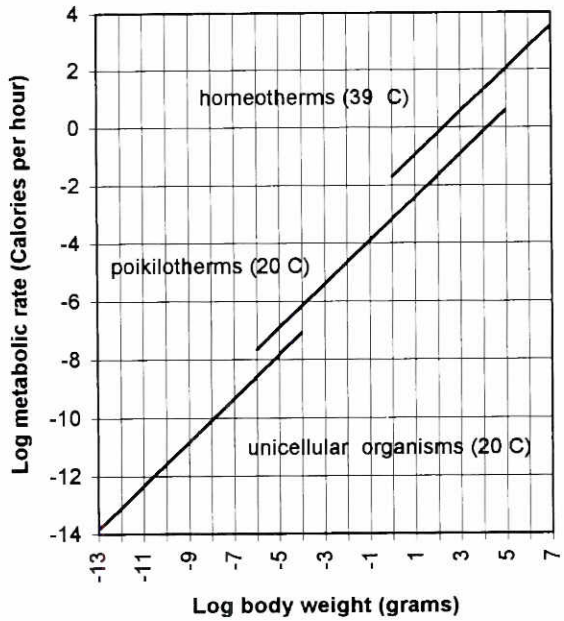

Fig. 1. Standard energy metabolism as a function of body weight based on Hemmingsen (1960).

$$\ln[\text{rate}(T1)/\text{rate}(T0)] = q\,[1/T0 - 1/T1],$$

where T is the absolute Kelvin temperature and q, while it is a function of temperature, is about 5×10^3 to 1×10^4 per degree Kelvin in this temperature range as determined by fitting the Arrnhenius equation to data for poikilotherms from Krogh (1914, 1916).

If the poikilotherm curve is corrected for temperature from 20°C to 39°C purely on the basis of chemical reaction rates, then it falls below the homeotherm curve by a factor between about 4 to 7, depending on which value of the Arrhenius constant is used. When the uncertainty in the constant, log k, is accounted for, the central 90% certainty range for the ratio of the metabolic rate between homeotherms and poikilotherms becomes very large indeed. Thus, it is ambiguous if the oxygen consumption is in fact inadequate to support the energy requirements of the embryo and its membranes. Experimental evidence, discussed below, indicates that by day 12.5 a cultured rat embryo is extremely sensitive to oxygen concentration indicating that by this gestational age anaerobic metabolism is not adequate to maintain it. Kleiber *et al.* (1943) also comment that the fetus does not need to maintain its own temperature, so maintenance energy demand is much lower than for homeotherms.

Köhler *et al.* (1972) have shown that rat embryos between day 8 and day 14 are increasing their dry weight and DNA content in such a way that the ratio of DNA to dry weight is about constant. That is, there is limited growth of individual cells, and size is increasing as a result of the increase in the numbers of cells. Throughout this period, the rate of cell division is slowing down. As the animal matures, cellular growth will replace cell division.

Energy supply

Energy metabolism of the preimplantation embryo is unusual (Brinster, 1974; Wales, 1975). In the first few cell divisions (early

cleavage stage) the energy substrate is pyruvate. Glucose metabolism is blocked. Brinster (1974) suggests that there is a defective malate shuttle system. He suggests that metabolic changes in the early embryo may be similar among many mammalian species in the first 5-7 days post conception. At the time of implantation, both pyruvate and glucose are about equally oxidized. He indicates that the ratio of lactate to CO_2 production increases from 1.1 for the unfertilized and fertilized egg to a maximum of 4.2 at the morula stage and down to 2.9 at the late blastocyst stage. This indicates the importance of anaerobic glycolysis for energy production in the early embryo.

Researches with whole embryo culture have shown that optimal growth requires different oxygen concentrations at different ages. New and Coppola (1970a,b) have shown that the best growth *in vitro* for rat embryos in circulating medium occurs at oxygen concentrations of 20% at days 9.5 and 10.5 post conception, at 95% at day 11.5, and with 97.5% at 2 atmospheres at day 12.5. By day 13.5, the *in-vitro* growth of rat embryos is very poor even at 2 atmospheres of oxygen. On the other side of the coin, however, extended exposure to 95% oxygen is detrimental to day 10.5 embryos (Shepard *et al.*, 1969).

New and Coppola (1970b) have also shown that even though day 12.5 embryos grow well under hyperbaric oxygen, the yolk sac, directly exposed to the culture medium perfused by the elevated oxygen concentration, is damaged. New and Coppola (1970b) showed that exposure to 3 atmospheres of oxygen will destroy the yolk sac. It is clear that, even if oxygen consumption in cultured embryos is less that would be the case *in vivo*, the more advanced the embryo's development, the more oxygen it needs. The allantoic placenta neither develops when younger embryos are cultured nor functions when older ones are cultured. This loss undoubtedly reduces the amount of oxygen delivered to the embryo. While the use of higher and higher oxygen concentrations in the perfusing gas for the older embryos may not completely substitute, the observed growth is not an unreasonable fraction of what would be observed *in vivo*.

The changing demand for oxygen is accompanied by the development of aerobic metabolism in the embryos as reflected by the increasing activity of the terminal electron transport system. Aksu *et al.* (1968) have shown the rapid increase of the specific activity of this system in the rat between days 10 and 11 of gestation. Tanimura and Shepard (1970) showed that glucose use decreases, carbon dioxide production increases and lactate production decreases per unit protein increase in the embryos from day 10 to day 12 of gestation, consistent with the observations of Aksu *et al.* (1968). Other oxidases show similar increase in activity from day 10 through day 14 of gestation and there are large increases in the number and structure of cristae in heart mitochondria in this period (Mackler *et al.*, 1971). Mackler *et al.* (1973) also have shown that when purified mitochondrial preparations from rat embryos from day 10-14 of gestation are used, there is little difference in their specific activity suggesting that the increase in total activity is due to the increasing amount of enzymes rather than to ongoing development of the enzyme activities.

Shepard *et al.* (1969) also observed that, when the culture gas was cycled at 10 minute intervals between 95% oxygen, air and nitrogen, the heart rate of day 10 $^1/_3$ embryos under air or nitrogen did not change significantly compared to the rate under 95% oxygen. When the experiment was repeated for day 11 and day 12 embryos, the heart rates did significantly decrease compared to the 95% reference value. When coupled to the observations of Aksu *et al.* (1968) on the rapid increase in the terminal electron transport system between day 10 and 11 of gestation, one may infer that it is in this period that the rapid conversion from anaerobic to aerobic metabolism is occurring. Concomitant with the increasing activity of the enzyme systems, the yolk sac circulation develops. By day 11 red blood cells are circulating throughout the yolk sac and embryo. As the hematocrit increases, so will the delivery rate of oxygen to embryonic cells.

Energy inhibitors – oxygen delivery

Rodent embryos in culture must rely on the yolk sac placenta since the allantoic placenta has not been able to be made functional *in vitro*. In mammals, oxygen is carried for the most part by hemoglobin in the red blood cells. In the early embryo, however, the heart starts beating and a circulation is established before the hematocrit becomes large enough to be useful as a blood transport vehicle.

In the case of the rat, before the production of red blood cells is established in early day 11 of gestation, the yolk sac and embryo are very small. Diffusion before the heart starts beating and diffusion and transport in physical solution in plasma after the heart beat is established are the dominant mechanisms for such oxygen as is required. Once the hematocrit reaches useful levels, the red blood cells are the major carriers of oxygen to the cells. After that time, exposing the embryo to a gas mixture containing high levels of carbon monoxide and eliminating the oxygen carrying capacity of the red blood cells will result in oxygen transport being limited to diffusion and simple physical solution.

For cultured embryos older than day 12, even hyperbaric oxygen is inadequate to completely provide the necessary oxygen by these purely physical means. However, Cockroft (1973) has shown that this problem can be circumvented for day 12.5 and day 13.5 rat embryos by opening the yolk sac, exteriorizing the embryo, maintaining the connection to its yolk sac, and allowing the oxygenated medium to contact the embryo directly. Using this technique, he was able to maintain embryos under 95% oxygen at 1 atmosphere of pressure during a culture period of 42 h.

Energy inhibitors – oxygen utilization

The utilization of oxygen in aerobic respiration is mediated by numerous enzymes that aid in the reaction of the metabolic breakdown products of glucose with oxygen and produce high energy phosphorus compounds. Carbon monoxide binds to the iron in cytochromes and prevents their participation in electron transport. Cytochrome P-450 is the terminal oxidase in many mixed function oxidase systems. Carbon monoxide binds rapidly with reduced P-450 and inhibits its functioning (Cooper *et al.*, 1970).

Inhibiting these respiratory enzymes and thereby reducing the utilization of oxygen should have the same effect as directly reducing the amount of oxygen available to the embryo. However, Chance *et al.* (1970) have shown that CO binds to most but not all cytochrome iron atoms. They show that the remaining functional cytochrome molecules are able to react both in their own chains and also branch out to other respiratory chains in which they are usually not active. They also describe another phenomenon called "cushioning" in which the respiratory chain can alter the redox

states of its components to maintain a steady flux of electron transport. They indicate that the oxidation-reduction level of the respiratory carriers might change considerably although the flux of electrons through the system might be relatively insensitive. If this is the case, then the effect of CO exposure will be limited to reduction in oxygen transport rather than any significant poisoning effect on the respiratory enzymes.

There is, however, an additional possible aspect to CO poisoning of respiratory enzymes. Based on the results of experiments on dogs and sheep late in gestation, Gurtner and Burns (1973) and Burns and Gurtner (1973) suggest that P-450 is a specific oxygen carrier in the lung and placenta. If this is the case, and if P-450 serves this purpose in rodents in the yolk-sac placenta as well as in the allantoic placenta, then even facilitated diffusion and solubility transport of oxygen will be negatively affected by CO. We do not, however, have any data as to when or whether placental P-450 is a significant facilitator of oxygen transfer for the rodent embryo.

Experimental observations *in vivo*

Tumasonis and Baker (1972) assayed the hearts of 17 day-old chick embryos that had been exposed during the previous 24 to 168 h to 425 ppm of carbon monoxide. They found that the heme-containing cytochrome oxidase was depressed, the non-heme-containing lactic dehydrogenase was not affected and there was hypertrophy of the hearts in the experimental chicks exposed to CO for 144 and 168 h.

Baker and Tumasonis (1972) found that hatched chicks incubated under 425 ppm CO were smaller than controls but otherwise normal. However, they found that from day 10-16 of incubation, the exposed embryos showed a statistically significant pattern of slightly greater weight than the controls although the individual day-to-day differences were not statistically significant. The decrease in size was not explicitly given in terms of weight for the hatched chicks.

The weights for the day 10-16 embryos were not identified as to wet or dry weights, but the values ranged from 2.6 grams at day 10 to 17.1 grams at day 16, suggesting that they were wet weights. In addition, they report that there was a "compensatory hypertrophy" of the extra-embryonic vascular plexus as well as vesiculations on the amnion. They did not report any observations of similar effects in the embryos themselves. If there were hypertrophy of the embryonic vasculature, blebs or edema, it would account for a slight wet weight increase of the exposed embryos compared to controls. By the time of hatching, the negative effect of the CO exposure could easily overcome the slight weight increases observed earlier. This idea is consistent with their observation of an abrupt increase in COHb concentration from day 14 (8.5%$\pm$1.7%) to days 15 (13.3%$\pm$2.5%) and 16 (16.4%$\pm$2.8%) of incubation. The negative effects of this very large increase of COHb just before the period of greatest absolute weight gain in the days just before hatching could easily dominate the slight differences observed earlier.

The negative effect of maternal smoking during gestation on birth weight of offspring has been suspected of being mediated by the increased carboxyhemoglobin in the blood. Astrup *et al.* (1972) exposed pregnant rabbits to CO at 90 and 180 ppm. The 90 ppm group and their matched controls were in their first pregnancy while the 180 ppm group and matched controls were in their second. The higher exposure gave a 20% decrease in birth weight and a neonatal mortality of 35% vs 0.9% in controls. The lower exposure resulted in a birth weight reduction of about 11% compared to controls and a neonatal mortality of 9.9% vs 4.5% in controls. Astrup *et al.* point out the first litters are usually smaller and have lower average birth weight than later litters. In addition, the control group for the lower exposure were kept in larger cages allowing greater freedom of movement for the pregnant control rabbits than for the exposed group. Consequently, the control dams may have been under lower stress than they would have been if their conditions were matched to the experimental group. This may have led to larger pups and hence the disparity in the birth weights of exposed and control offspring.

Astrup *et al.* (1972) also describe data indicating that in pregnant women smokers, birth weight was negatively correlated with COHb percentage in the maternal blood at a statistically significant level ($p<0.05$). The carboxyhemoglobin concentrations they observed ranged from a mean value of 0.87% for non-smoking women to a mean value of 1.92% in their smoking cohort. The COHb concentrations in their smoking cohort ranged up to 10% (1 of 824 women in cohort), with 5% of the cohort above 5% COHb, while in their non-smoking cohort the values ranged up to 8% (1 of 884 women) with 5% of the cohort above about 2.5%.

Longo (1977) measured the COHb concentration in pregnant women and fetuses when the mother had smoked 1 or more cigarettes 1 h or less before delivery. He quotes values of 6.2% and 7.6% respectively. For exposure to an effective steady state concentration of 30 ppm (partial pressure 0.024 mm Hg), Longo (1977) indicates the maternal COHb concentration would be about 5% with the fetal level about 5.5%.

Longo's measurements are supported by the results of Soothill *et al.* (1996). They measured the fetal COHb in fetal cord blood for fetuses between 18 and 35 weeks of pregnancy. For non-smoking mothers they report an average concentration of 2.9% while for smoking mothers the concentration was 6.2%. They found no statistical correlation between fetal age and COHb concentrations. However, they did observe a slight increase in total fetal hemoglobin concentration in the fetuses of mothers who smoked. They point out that the increased production and quicker turnover of hemoglobin would help to dilute and clear the COHb faster than normal which would tend toward some mitigation of the effects of carbon monoxide in the fetus.

Osborne *et al.* (1956) analyzed the composition of cigarette smoke. Their results indicate that the dose of CO per puff is about 1.4 ml (STP). If we consider a heavy smoker as consuming two packs per day (40 cigarettes) and taking 10 puffs per cigarette, the total daily dose would be 560 ml of CO. Assuming it takes about 5 min to smoke a cigarette and consumption is uniform over a 16 hour waking day, the interval between cigarettes would be about 20 min.

The International Commission on Radiological Protection report on reference man (ICRP 1975), indicates that for an adult woman under light activity, the respiration rate is 19 litres per min. If it takes 5 min to smoke a cigarette, during which time an individual inhales about 14 ml of CO and breaths about 95,000 ml of air, the average concentration while smoking would be about 150 ppm in the inspired air. For a female heavy smoker, the average CO concentration in the inspired air over a 16 hour period would be about 30 ppm (0.024 mm Hg) and over an entire 24 hour day would be about 20 ppm (0.015 mm Hg).

Godin and Shephard (1972) show that there is an initial very rapid (about 1 min) uptake of CO by pulmonary blood followed by

a slower approach to systemic equilibrium (about 5 min). They indicate that the clearance rate of COHb from the blood is very slow for the first 15 to 20 min following cessation of exposure after which the elimination of the increment over the intrinsic level (control COHb: 1%) proceeds with a half time of about 4 h.

During an 8 hour sleep period, the COHb would clear from the blood so that on waking the average COHb concentration would be approximately 2/3 of the value at the beginning of the sleep period assuming the Godin and Shephard (1972) clearance rates. Taking Longo's (1977) value of 5% COHb at an exposure of 30 ppm of CO, for 2/3 of the day, the blood concentration would be about 5%, and for 1/3 of the day (sleep period) the average concentration would be about 1/3 less. Assuming that at low concentrations of CO, the fetal concentration remains at about 10% above that of the mother, the overall daily average COHb concentration would be equivalent to about 4.4% in the mother and about 4.8% in the fetus. While these levels are undoubtedly undesirable, there is still over 95% of the hemoglobin uncombined with CO and able to function as an oxygen carrier.

When pregnant rats were exposed to smoke from tobacco cigarettes or lettuce cigarettes with and without added nicotine and compared to unexposed controls fed varying amounts of a standard diet, the effect of CO was interpreted differently (Younoszai *et al.*, 1969). Younoszai *et al.* noted that rats exposed to either tobacco or lettuce smoke ate less than controls. Fetal weight loss of lettuce smoked rats with or without nicotine correlated with fetal weight loss of food restricted controls on an equal food consumption basis. The lettuce leaf plus nicotine smoked dams had slightly smaller pups and also ate slightly less than in the case of those smoked with lettuce alone. Tobacco cigarette exposed rats had lowest weight pups and ate the least. They concluded that CO had no particular direct effect on fetal growth and weight gain, and that all of the effect seemed to be mediated through reduction in maternal food consumption.

A contrary result was subsequently observed by Haworth and Ford (1972). In their experiment, pregnant rats whose food intake was restricted to match that in pregnant rats exposed to cigarette smoke showed fetal weights which were not significantly different from unsmoked controls fed *ad libitum*. The fetal body weights in the tobacco smoke exposed rats were significantly smaller than for either the controls or the unsmoked group that was matched for food consumption with the smoke exposed group.

In Astrup's (1972) experiment with rabbits, their food consumption was not reported. Similarly differences in diet between the smoking and non-smoking women were not reported. Thus a direct comparison with the results of Younoszai *et al.* (1969) is not possible. However, appetite suppression due to cigarette smoking has the status of folklore.

Experimental observations *in vitro*

Rat heart muscle cells cultured under 20% oxygen showed an interesting response to carbon monoxide (Wenzel and Brenner 1973). The beat rate increased in proportion to increasing CO. In the presence of CO, the beat rate decreased with decreasing oxygen concentration. Cell growth also decreased under CO with a greater decrease with decreasing oxygen concentration.

Robkin *et al.* (1976) explanted days 10.5, 11 and 12 rat embryos into a bubble-driven circulator (New, 1967) with various mixtures of oxygen and carbon monoxide. Day 10.5 embryos were gassed with 95%air+5% CO_2 while day 11 and 12 embryos were gassed with 95%O_2+5%CO_2 mixtures. CO at 0.04, 5 and 10% by volume was added to these gas mixtures for the test embryos. Over an observation period of 2 h there was no effect on the heart rate of the embryos under any of these CO mixtures so long as the appropriate oxygen concentration was supplied. In a similar experiment, Shepard *et al.* (1969) supplied cultured rat embryos of gestation ages 10 $^1/_3$, 11, and 12 days with a gas mixture of 95% O_2+5% CO_2 followed by a gas mixture of either 95% air+5% CO_2 or 99.5% N_2. In this short term (10 min) exposure sequence, the response of the embryonic heart rate was monitored. The 10 $^1/_3$ day embryos were relatively insensitive to any of these perfusion mixtures while the day 11 and day 12 embryos showed prompt decrease in their heart rates with exposure to nitrogen giving a somewhat greater decrease than exposure to 95% air. Nitrogen exposure also stopped the yolk sac circulation in these older embryos. They also observed that exposure of the 10 1/3 day embryos to 95% oxygen for 12 h or more was detrimental.

In a study with rat embryos explanted at day 11 and cultured for 18 h in the roller tube culture system of New *et al.* (1973), Robkin and Cockroft (1978) examined the effect of carbon monoxide on glucose metabolism and growth. They compared growth, development, glucose consumption and lactate production for embryos exposed to gas mixtures of 85% O_2+10%N_2+5%CO_2, 40%O_2+55%N_2+5%CO_2, 85%O_2+10%CO+5%CO_2 and to a mixture of 99% of 40%O_2+55%N_2+5%CO_2 and 1% CO.

After 18 h of culture, there was no significant difference ($p>0.05$) in the number of somites or crown-rump length between the higher oxygen mixture and the higher oxygen mixture plus 10% carbon monoxide or between the lower oxygen mixture and the lower oxygen mixture plus 1% CO.

Rat embryos at this stage of gestation are increasing their protein content exponentially (Robkin and Cockroft, 1978). Their growth rate can be characterized by the fractional increase in their protein per unit time. By this criterion, there was a significant decrease in the embryo's growth rate compared to controls during the 18 hour culture with CO under the higher oxygen mixture ($p<0.008$). When the control embryos were cultured under 40% oxygen, and the experimental embryos under 40% O_2 + 1% CO, the decrease in growth rate was not significant ($p>0.05$). There was significant increase in glucose consumption ($p<0.05$) and lactate production ($p<0.008$) with the added CO when compared to controls at the higher oxygen level but not when the controls at the lower oxygen level were compared with embryos cultured under the lower level with the smaller percentage addition of CO. The ratio of lactate produced to glucose consumed was also significantly ($p<0.008$) increased due to the addition of CO with the higher oxygen level test but not with the lower level test.

When the physiological parameters were compared between the case of 85% oxygen with 10% CO and the case of 40% oxygen without CO, there was no significant difference in the growth rate but the lower oxygen level resulted in a significantly greater consumption of glucose and production of lactate. In addition, the ratio of lactate production to glucose consumption was significantly higher for the 40% O_2 case compared to the 85% O_2 + 10% CO case.

In the absence of carbon monoxide, when the high oxygen culture was compared to the lower oxygen culture similar results were observed. When the oxygen level was reduced, the rate of protein increase was smaller ($p<0.04$), lactate production increased

($p<0.002$) as did glucose consumption ($p<0.001$). The ratio of lactate production to glucose consumption increased significantly ($p<0.001$).

Summary and Discussion

Mammals are homeotherms and expend considerable energy maintaining their body temperatures. The temperature of a mammalian embryo on the other hand is maintained by the mother and the embryo can devote its metabolic energy to growth and development. The mammalian embryo is acting as a poikilotherm and its energy needs are thus considerably less than if it were a comparably sized homeotherm.

The energy requirements of the preimplantation rat embryo is generated by anaerobic metabolism. As it grows, aerobic metabolism develops. Tanimura and Shepard (1970) have shown that at least up to the 35 somite stage (day 12) the rat embryo is relying on a combination of aerobic and anaerobic metabolism.

The rat embryo has two placental circulations to rely on. The first to develop is the yolk sac circulation at about day 11 followed in a couple of days by the allantoic placenta. The allantoic placenta is much more efficient and will carry the oxygen load in the latter part of gestation. In culture, unfortunately, it has not yet been possible to achieve a functioning allantoic placenta, and the increasing demand for oxygen has been met to the extent possible with the yolk sac placenta using various ingenious methods.

Rat embryos at days 9.5 and 10.5 of gestation are small enough that with 20% oxygen perfusing the culture medium the embryos grow remarkably well. By day 11.5, 20% oxygen is inadequate. The first red blood cells appear in the yolk sac vessels at about day 10.5 of gestation at about the 15 somite stage. By day 11.5 of gestation the embryo has considerable numbers of red blood cells circulating between it and its yolk sac. The oxygen carrying capacity of the circulation is increased, although the hematocrit may be low (Robkin and Cockroft, 1978). The culture gas now requires 95% oxygen. Oxygen dissolved in the culture medium diffuses across the yolk sac surface to be transported to the embryo both by simple solution and by the circulating red cells. With this concentration of oxygen in a 24 hour culture, the embryo grows well but at a rate somewhat less than that *in vivo*. By day 12.5, the lack of a functioning allantoic placenta is apparent. The culture can continue with the use a culture gas containing 97.5% oxygen at 2 atmospheres of pressure. By day 13.5, it is necessary to open the yolk sac without damaging its circulation so that the culture medium can directly contact both the yolk sac and the embryo (Cockroft, 1973). Even with 95% oxygen at 1 atmosphere, this allows enough oxygen transfer to permit the culture period to extend nearly two additional days.

The addition of carbon monoxide to the gas perfusing the culture medium for early rat embryos has a much smaller effect than decreasing the oxygen concentration. Carbon monoxide reduces the efficiency of RBCs for oxygen transport and also may inhibit facilitated oxygen transport through the placenta and somewhat inhibit the enzymes of oxidative glycolysis. The evidence indicates, however, that the primary effect comes from reduction of the oxygen available to the embryo. In culture at 11.5 days of gestation, the contribution of the red blood cells to oxygen delivery is not dominant and the embryo is producing energy from both aerobic and anaerobic glycolysis. Carbon monoxide appears to be a relatively mild toxicant until the embryo is much larger, is depending much more on transport of oxygen by red blood cells, and the fraction of required metabolic energy produced by anaerobic metabolism has become quite small.

In the case of cultured embryos at day 10.5 and earlier, the small size of their yolk sac and themselves may allow the oxygen concentration in their cells to be abnormally high and directly toxic when the oxygen concentration in the culture medium is high. This is underlined by the detrimental effect of high oxygen concentration on the yolk sac.

By day 11.5, the transition to aerobic metabolism is well under way. For embryos of this age, the increase in cellular oxygen consumption plus the increase in the gas diffusion path from the culture medium through the larger yolk sac to the larger embryo itself requires a high oxygen concentration in the culture medium to maintain even an approximately normal oxygen concentration in their cells. By the time that the conceptus has grown beyond 13 days, even with a well developed yolk sac circulation and hyperbaric oxygen concentrations perfusing the culture medium, the absence of a functioning allantoic placenta means that the surface area and diffusion capacity for gas transfer is inadequate to keep up with the oxygen demand of the embryonic tissues. In addition, the directly toxic effect of oxygen on the yolk sac is undoubtedly interfering with oxygen transfer both by diffusion and by solution and is a significant inhibitor of embryonic growth.

The effect from smoking during gestation may be either by the concomitant reduction in food intake or a more direct toxic effect from some components in the smoke. Carbon monoxide does not seem to be the culprit. This would be supported by the consideration that even a heavy smoker and her fetus still have over 95% of their hemoglobin functioning as an oxygen carrier. If the mother can smoke without herself suffering a significant immediate hypoxia, then there is probably sufficient oxygen being delivered to the fetus to sustain it. The possible mitigating effect of a compensatory increase in fetal hematocrit in response to any hypoxia must also be considered.

The question remains as to whether total food reduction in pregnant mammals has a significant effect. If there are enough essential nutrients provided, such as total protein, vitamins and minerals, the reduction in total calorie intake may be only a secondary contributor to reduced fetal weight. Perhaps maternal fat stores can make up for any calorie shortfall. The main contributor to fetal weight reduction in smoking mothers may well be one or more of the many other toxic components of cigarette smoke. Food intake and/or smoking toxicants other than carbon monoxide need to be further investigated as an alternative to maternal COHb levels as a correlator of reduced birth weight and other detrimental effects in humans.

The bottom line seems to be that for early embryos, before their demand for oxygen can only be satisfied by transport via the red blood cells, carbon monoxide is not a very important toxicant. There may be some reduction in cellular energy production and oxygen transfer due to some enzyme inhibition and the loss of some RBC function, but the embryo appears to be able to compensate considerably.

Comparison of the stages of development between the rat and the human (Altman and Dittmer, 1972) indicates that, based on the Witschi stage and the somite count, day 11 of gestation in the rat is equivalent to day 28 in the human and day 12.5 in the rat equivalent to day 37 in the human. Humans do not have a yolk sac placenta, but if the switch from anaerobic to aerobic metabolism is

correlated with the stage of development, then the risk to the human embryo from carbon monoxide exposure will be much greater after this period. Indeed, MacArthur and Knox (1988) have shown that women who stop smoking before week 16 of pregnancy have babies whose weights are essentially the same as non-smokers. Their study group were only moderate smokers (average of 13-14 cigarettes per day), but their results reinforce the conclusion that the toxic effects of smoking are much more significant in the second half of pregnancy.

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