EFFECTS OF DIFFERENT OXYGEN CONCENTRATIONS ON THE DEVELOPMENT OF RAT EMBRYOS IN CULTURE

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Summary. Rat embryos explanted with their membranes at 9½, 10½, and 11½ days' gestation have been grown in homologous serum under different conditions of oxygenation. In circulating serum, maximum growth of the younger embryos was obtained in 5% CO₂ in air and of the older embryos in 5% CO₂ in 95% O₂. In watch glass cultures, the younger embryos developed well in 5% CO₂ in 60 to 95% O₂, but growth of the older embryos could only be obtained in hyperbaric oxygen. The yolk sac of the older embryos continued to expand normally under oxygen conditions which were inadequate for growth of the embryos, but the heart and blood circulation functioned best under conditions which were optimal for embryonic growth. Although the proportion of O₂ required in the gas phase varied greatly with the type of culture and the stage of development of the embryo, the results could be explained on the assumption that embryonic tissues at all stages require an O₂ pressure similar to that provided by blood in equilibrium with air.

INTRODUCTION

Explanted rat embryos of post-implantation stages can be grown in homologous serum, either in watch glass cultures (New, 1966b) or in circulating serum (New, 1967). These methods are of value in studying mammalian embryo physiology and in investigating the causes of embryonic malformations (e.g. Turbow, 1966). Berry (1968) has recently examined the growth rates of rat embryos in culture, and Givelber & Di Paolo (1968) have successfully used the circulator method for growing post-implantation hamster embryos.

A critical requirement of embryos in culture is a carefully regulated O₂ supply. This can be varied in three ways: (1) by altering the proportion of O₂ in the gas phase of the culture vessel, (2) by increasing the pressure of the gas phase—hyperbaric oxygen, (3) by altering the rate of flow of oxygenated medium round the embryo. Previous work has suggested that there might be an optimum O₂ level for each stage of embryonic development. This paper describes the effects of variations of O₂ supply on rat embryos explanted at different stages.

MATERIALS AND METHODS

Embryos were obtained from the Hooded and Wistar strains of rats, and were explanted with their embryonic membranes by a method similar to that described previously (New, 1966a, 1970). Each conceptus was dissected free of the surrounding decidua in Tyrode saline and, before the conceptus was placed in the culture vessel, the Reichert membrane was torn open.

The culture medium used throughout was homologous serum containing 50 $\mu\text{g/ml}$ streptomycin. The serum was obtained from the dorsal aorta of rats anaesthetized with ether and it was stored for periods of up to 1 week at 1 to 4° C or for longer periods at -10° C.

Some of the embryos were grown in watch glasses of serum, housed in Petri dishes lined with wet gauze (New, 1966b) as in the standard method for organ culture. Others were grown in small tubular chambers through which serum was circulated. Except where stated otherwise, these chambers were square in cross section, 7 $\times$ 7 mm internal.

The apparatus for maintaining circulation of the serum has been described previously (New, 1967). The only modification introduced was an improvement in the method of anchoring the embryos within the embryo chamber. Previously the embryos were attached by means of the opened Reichert membrane to a strip of untreated rayon fabric stuck to a piece of glass coverslip with a few drops of acetone. It has been found more reliable to soak the rayon before use in thick collagen solution and then to allow it to dry on to the coverslip. Not only does this improve the adhesion between rayon and coverslip, but when the Reichert membrane of the explant is pressed against the treated rayon it also attaches more firmly.

The gas in which the embryos were grown always contained 5% CO₂ although the proportion of O₂ was varied. The watch glass cultures were incubated in sealed chambers containing the required gas mixture. The serum in the 'circulators' was oxygenated by bubbling the gas through it.

All cultures were terminated after 40 to 50 hr incubation. The main criteria used in estimating the development and duration of survival of the embryos in culture were the rate of heart beat, persistence of the blood circulation, and the final number of somites and protein content. Protein determinations were always made on the embryos alone after dissecting them free of the embryonic membranes; the embryos were digested in NaOH and the protein was estimated by comparison with standard solutions of bovine plasma albumin, using the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Embryos explanted at 9½ days (primitive streak to head-fold)

Table 1 summarizes the results from embryos explanted into circulators at 9½ days, and grown in serum equilibrated with three different O₂ concentrations and circulated at various rates of flow. Each treatment was given to a group of four to eight embryos, care being taken that the embryos in the different groups were comparable as regards stage of development at the time of

explantation. Where the O_2 concentration or rate of flow was altered during the culture period—as in Group 3—the change was made after the first 24 hr. After 48 hr in culture, the embryos were assessed for somite formation and protein content.

Even at very slow rates of flow (Groups 10 and 11), 95% O_2 proved harmful to the explants. The yolk sacs expanded only to about 1.5 to 2.0 mm diameter, no blood circulation was established, and embryonic development was limited to a small knob of tissue in which a few somites could be counted only with some difficulty. However, 95% O_2 gave good results if introduced after 24 hr (Group 9) when the embryos had grown to ten or more somites. The embryos

TABLE 1
DEVELOPMENT OF EMBRYOS EXPLANTED AT PRIMITIVE
STREAK TO HEAD-FOLD STAGES AND GROWN IN CIRCULATING
SERUM

Group no.	Oxygen concentration	Flow rate (ml/min)	Somites formed	Protein synthesized (μ g)
1	5%	1	14	39
2	5%	10	15	42
3	5%→20%	1→10	13	50
4	20%	0.2	17	75
5	20%	0.8	16	66
6	20%	4	22	83
7	20%	1→10	21	90
8	20%	10	19	69
9	20%→95%	1	19	79
10	95%	0.3	6	<20
11	95%	1	2	<20

developed well in all the cultures with 20% O_2 (Groups 4 to 8) and much better than in those with 5% O_2 (Groups 1 and 2) even when this was replaced by 20% O_2 after the first 24 hr (Group 3). Of the embryos grown in 20% O_2 , the best results were obtained in serum flowing at 4 ml/min (Group 6) and 1 ml/min increased to 10 ml/min (Group 7), but the differences are too small to be certain from this number of embryos that there is here any indication of optimum rates of flow.

In serum equilibrated with 20% O_2 , the yolk sacs expand to 3 to 4 mm diameter and the better embryos develop a good blood circulation with a heart rate of 130 to 140 beats per min. Those explanted at head-fold stages (conceptus length >1.5 mm) grow much better than younger stages and may develop up to twenty-eight somites.

Comparison was made of embryos grown in circulators with those grown in watch glasses containing 1 ml serum. The explants from 9½-day pregnant rats sink in serum and their respiration is therefore affected by the depth of the serum in the watch glass. Some of the explants were supported on a strip of coverslip in the watch glass so that the embryo lay just below the surface of the

	Initial condition	Final condition	Oxygen	Somites	Protein (μg)
(a)			$\left\{ \begin{array}{l} 20\% \\ 95\% \end{array} \right.$	$\left\{ \begin{array}{l} 8 \\ 18 \end{array} \right.$	$\left\{ \begin{array}{l} 30 \\ 62 \end{array} \right.$
(b)			$\left\{ \begin{array}{l} 20\% \\ 60\% \\ 95\% \end{array} \right.$	$\left\{ \begin{array}{l} 12 \\ 19 \\ 0 \end{array} \right.$	$\left\{ \begin{array}{l} 46 \\ 65 \\ <20 \end{array} \right.$
(c)	In circulators (flow 2 to 10 ml/min)		20%	19	65

TEXT-FIG. 1. Development of embryos explanted at primitive streak to head-fold stages and grown (a) attached to the bottom of a watch glass of serum, (b) supported near the surface of a watch glass of serum, and (c) in circulating serum.

TEXT-FIG. 2. Development as indicated by somites formed (open columns) and growth as indicated by final protein content (stippled columns) of embryos explanted at 10½ days and grown in circulating serum.

serum throughout the culture period. Others were placed in the bottom of the watch glass so that initially the embryo lay under about 2 mm depth of serum but was gradually raised up through it as the yolk sac expanded. Thirty embryos were divided into six groups for different treatments and the results are shown in Text-fig. 1. The best results were obtained from the supported embryos in 60% O₂, the more deeply submerged embryos in 95% O₂ and those in circulating serum equilibrated with 20% O₂; the results from these three groups were very similar.

Embryos explanted at 10½ days (six to fifteen somites) and 11½ days (twenty-two to twenty-eight somites)

Text-figs. 2 and 3 summarize the results of five experiments in which embryos explanted at 10½ and 11½ days were grown under different conditions of oxygenation. Three or four litters were used for each experiment but the embryos of

TEXT-FIG. 3. Somites formed (open columns) and final protein content (stippled columns) of embryos explanted at 11½ days and grown in circulating serum.

each litter were divided among the different treatments so as to eliminate initial differences between litters. Each rectangle in the histograms represents the mean of determinations made on a group of embryos receiving a particular treatment.

In Exp. I, the embryos were explanted at seven to fifteen somites (20 to 60 μg protein), and were divided into three groups each of twelve embryos. All were cultured in 95% O₂. Most growth and development was shown by the embryos in serum flowing at 1 ml/min. The final protein content of these was significantly

greater than that formed either by the embryos in watch glasses ($P < 0.01$) or those in serum flowing at 10 ml/min ($P < 0.01$).

In Exp. II, the embryos were explanted at six to eleven somites, and were divided into three groups each of six embryos. Final protein was significantly greater ($P < 0.01$) in 95% O_2 at 1 ml/min than in 20% O_2 at 10 ml/min, and it was significantly greater ($P < 0.01$) in 20% O_2 at 10 ml/min than in 95% O_2 at 10 ml/min.

In Exp. III, the embryos were explanted at nine to fourteen somites, and were divided into four groups each of four to six embryos. Final protein was similar in 95% O_2 at 1 ml/min and in 20% O_2 at 10 to 20 ml/min, but significantly greater in both than in 20% O_2 at 1 ml/min ($P < 0.001$).

TABLE 2
GROWTH OF YOLK SAC AND EMBRYO UNDER DIFFERENT CONDITIONS OF OXYGENATION

Treatment	No. of embryos	Yolk sac diameter (mm) mean $\pm$ S.E.	Embryo protein (μ g) mean $\pm$ S.E.
A { 95% O_2 1 ml/min	8	6.4 $\pm$ 0.1	796 $\pm$ 69
95% O_2 15 to 20 ml/min	8	6.4 $\pm$ 0.2	1306 $\pm$ 123
B { 20% O_2	12	6.9 $\pm$ 0.2	400 $\pm$ 40
95% O_2	12	6.4 $\pm$ 0.2	1010 $\pm$ 102

In Exp. IV, the embryos were explanted at twenty-two to twenty-eight somites (150 to 300 μ g protein) and divided into four groups each of six embryos. All were cultured in 95% O_2 . Growth of the embryos in watch glasses was negligible. Of the embryos in circulators, final protein was very significantly greater ($P < 0.001$) in those in serum flowing at 15 ml/min than in those in serum flowing at 1 ml/min.

In Exp. V, the embryos were explanted at twenty-two to twenty-eight somites and divided into four groups each of eight embryos. The two groups in serum flowing at 15 ml/min were grown in the standard 7 $\times$ 7-mm embryo chambers. These chambers proved unsatisfactory for much higher rates of flow because of the excessive agitation and distortion of the yolk sacs. The two groups in serum flowing at 75 ml/min were therefore grown in slightly larger embryo chambers as shown in Text-fig. 4. These were cylindrical and 10 mm in diameter, and the embryos were supported in the chamber on rayon raised from the floor of the chamber on a wire frame, so that the serum flowed under as well as over and around the explants. However, the histogram shows that both groups of embryos in 95% O_2 attained similar stages of growth and development and the final protein was significantly greater ($P < 0.001$) than in either group in 20% O_2 .

Heart and blood circulation

The embryonic and yolk sac blood circulations developed well in nearly all the explanted embryos, except those cultured under the most harmful con-

ditions of oxygen lack or excess. In the $10\frac{1}{2}$ - and $11\frac{1}{2}$ -day embryos the circulation was maintained for 30 hr or more but had usually failed by 40 hr. In the $9\frac{1}{2}$ -day embryos, a good circulation was often present 40 hr after explantation but it rarely persisted beyond 48 hr.

The heart rate of embryos explanted at $9\frac{1}{2}$ and $10\frac{1}{2}$ days was usually 80 to 90 beats per min at the time of onset of the blood circulation (about the ten-somite stage) rising to 120 to 150 as development progressed. Heart rates of embryos explanted at $11\frac{1}{2}$ days usually attained 140 to 170 beats per min, occasionally increasing to 190.

When growth was hindered by an excess or shortage of O_2 , the blood circulation and heart rate were also affected. For example, $10\frac{1}{2}$ -day embryos which

TEXT-FIG. 4. Two types of embryo chamber used in circulators.

were over-oxygenated by growing them in rapidly flowing serum equilibrated with 95% O_2 rarely developed a heart rate of more than 100 beats per min. On the other hand, the $11\frac{1}{2}$ -day embryos growing in 20% O_2 (Text-fig. 3) had no yolk sac blood circulation after 18 hr and an average heart rate of only 65 beats per min, compared with the well developed circulation and heart rate of 150 to 170 shown by those in 95% O_2 .

In vivo, the allantoic circulation is established at about the twenty-somite stage and the embryos explanted after this stage maintained the allantoic circulation in culture. Younger embryos failed to develop an allantoic circulation and often formed a large abnormal vessel which bypassed the allantoic vessels, as described previously (New, 1967).

Growth of the yolk sac

Culture conditions that favoured growth of the younger embryos also favoured growth of the yolk sac. In the $11\frac{1}{2}$ -day explants, however, embryo and yolk sac responded very differently to variations in O_2 supply. This is shown in Table 2. Each part of the table compares the growth obtained under two levels of oxygenation, and litters were divided equally between the two treatments to ensure that the embryos were comparable. All the embryos were at twenty-two- to twenty-eight-somite stages when explanted.

Table 2A compares growth in serum circulated at 1 ml/min with that in serum circulated at 15 to 20 ml/min. All the serum was equilibrated with 95% O_2 . The results show that the faster flowing serum supported much more

growth of the embryos than the slower, but there was no difference in the size attained by the yolk sacs.

Table 2B compares growth in circulated (15 to 75 ml/min) serum equilibrated with 20% O₂ with that in serum equilibrated with 95% O₂. Again the embryos, but not the yolk sacs, grew much more under the conditions of higher oxygenation. The average yolk sac diameter was in fact slightly, but significantly ($P < 0.05$), greater in the 20% O₂ than in the 95% O₂.

Hyperbaric oxygen

A few trials were made with embryos cultured in hyperbaric oxygen. Twenty-four 11½-day embryos were placed in serum in small cylindrical dishes and either allowed to float in the serum or attached to rayon on the bottom of the dish and covered with serum to depths varying from 0 to 5 mm. Gas chambers containing the cultures were filled with 95% O₂-5% CO₂ and then extra oxygen was added to give a gauge reading of 15 lb/in.² (= total pressure of 2 atmospheres) or 30 lb/in.² (= 3 atmospheres).

The results showed that appreciable growth (up to thirty-eight somites and with twice the protein content at explantation) can be obtained from 11½-day embryos grown in hyperbaric oxygen as compared with the negligible growth of these embryos in watch glass cultures in 95% O₂-5% CO₂ at normal pressure. But controls grown in circulating serum equilibrated with 95% O₂-5% CO₂ developed further, up to forty-five somites and with three to four times the protein content at explantation.

Five embryos explanted at 12½ days (thirty-six to forty somites) and cultured under the same conditions in oxygen at 2 atmospheres showed no growth or development.

DISCUSSION

The work that has been described leads to the following main conclusions:

(1) Embryos explanted at head-fold stage grow to twenty to twenty-five somites in watch glass cultures in 60 to 95% O₂ or in circulating serum equilibrated with 20% O₂. Poor results are obtained in watch glass cultures in 20% O₂, or in circulating serum equilibrated with 5% or 95% O₂.

(2) Embryos explanted at about the ten-somite stage grow to twenty-five to thirty somites in watch glass cultures in 60 to 95% O₂, or to thirty to thirty-five somites in circulating serum equilibrated with 20% or 95% O₂. The rate of flow of serum in the circulators must be rapid with 20% O₂, slow with 95% O₂.

(3) Embryos explanted at about the twenty-five-somite stage grow to forty to forty-five somites in rapidly circulated serum equilibrated with 95% O₂. Little growth is obtained in watch glass cultures or in circulating serum equilibrated with 20% O₂. Hyperbaric oxygen improves the results in static serum but the amount of growth is much less than in circulating serum.

(4) The heart and blood circulation function best in the same oxygen conditions that promote most embryonic growth.

(5) Yolk sac expansion continues at oxygen concentrations below those necessary for embryonic growth.

It is clear that rat embryos can easily be killed by too little or too much O_2 and that the optimum concentration for growth in culture varies widely with the stage of development of the embryos. It is well known that major abnormalities of development *in vivo* can result from abnormal oxygen conditions (Zarrow, 1961), and the simplest explanation for the apparently varied oxygen requirements in culture may be that the embryo requires in its tissues an oxygen pressure similar to that provided by blood in equilibrium with air. Thus, in circulating serum, the younger embryos grow best in 20% O_2 but, in static serum, require higher concentrations of O_2 in the gas chamber to allow for the gradient established between gas phase and embryo. For embryos of more than twenty somites, there is a shortage of respiratory surfaces in culture because they lack the allantoic circulation present *in vivo*. To compensate for this, the O_2 in the serum surrounding the yolk sac must be raised (to 95% for maximum results) so that sufficient will be carried by the yolk sac blood circulation, and diffuse through the yolk sac and amniotic fluids, to maintain the optimal level in the embryo.

The yolk sac itself, which is in direct contact with the nutrient serum, expands normally in 20% O_2 at all stages from primitive streak to forty-five somites. It is also surprisingly tolerant, in the later stages, of serum equilibrated with 95% O_2 and it is this tolerance that makes possible the growth of embryos beyond the thirty-somite stage.

No embryo develops for long unless the heart and blood circulation are functioning well. It seems likely that the failure of the largest embryos to develop beyond forty-five somites may be caused primarily by slowing of the heart beat resulting from O_2 shortage. Shepard, Tanimura, Robkin & Bass (personal communication) have observed that, if the gas phase of cultures containing twenty- to thirty-somite embryos in circulating medium is changed from 95% O_2 to 20% O_2 , the heart rate falls to low values within a few minutes.

The problem of supplying sufficient O_2 in culture to organs and tissues of more than a millimetre or so in thickness has been studied by several workers in recent years (e.g. Trowell, 1961; Moscona, Trowell & Willmer, 1965). Increasing the proportion and pressure of the O_2 in the gas phase has been recognized as a useful method, but the value of flowing media has been more uncertain. Results have often been poor, and it has been suggested that flowing media may leach out essential metabolites. Our results with rat embryos have emphasized both the heavy demand for O_2 made by the larger embryos and the importance of flowing medium as a means of supplying it. If any harmful leaching occurred in our flow cultures, its effects must have been small compared with the advantages gained from the increased O_2 supply.

As yet, no post-implantation rodent embryos of stages younger than primitive streak or older than forty-five somites have been successfully grown in culture. Some promising results with rabbit embryos of implantation stages have been obtained by Glenister (1966) and Daniel (1970). Krishnan & Daniel (1967) have shown that in the rabbit a specific uterine protein, blastokinin, acts as an important stimulus to the earlier stages of embryonic development, and it seems likely that substances with a similar function will be identified in rats, mice and other rodents. For the larger embryos, the limiting factor appears to be rate of

O₂ supply. The use of hyperbaric oxygen and of flowing medium have each extended embryonic development well beyond the more conventional culture systems but the limits of these two methods alone seem now to have been reached. There remains the possibility of employing them in combination using flowing medium inside a pressure chamber and this is now being investigated.

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