Int. J. Dev. Biol. 41: 127-137 (1997)

A comparative and historical review of culture methods for vertebrates

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Introduction

Nature has evolved many stratagems by which organisms can reproduce themselves. The earliest animals lived in the sea, and when multicellular animals developed, their internal body fluids reflected the composition of their environment – salt water. Colonisation of the land, and indeed of fresh water, required that animals were able to regulate their internal environment. However, this posed further complications in that reproduction required a degree of replication of the ancestral marine environment. Different animal groups have devised various ingenious ways of coping with these problems. Amphibians are for the most part tied to water for their reproduction, although some species are able to take advantage of minute quantities of water, or its infrequent availability, even to the extent of laying their eggs in an aqueous jelly high amongst the leaves of trees, or in burrows in desert regions. Furthermore, some amphibians have astonishingly rapid development, to take advantage of what little rainfall there is. In other amphibian species, one or other of the parents carries the developing embryos, thereby protecting them from desiccation and predation (Young, 1962). Development of a more or less self-contained (i.e. *cleidoic* - literally 'closed box') egg in reptiles and birds meant that the liquid milieu and nutrients needed for early development could be provided independent of the surroundings, and by the time of hatching the organism would be able to regulate its own internal environment and resist the hostile dehydrating effects of dry land. Some groups further improved the protection of the young by retaining their fertilized and developing eggs for a time in the mother's body, so that a degree of viviparity was attained (ovoviviparity), but still it was necessary to invest the egg with all the nutrients required to develop to a stage where free living was possible.

With the evolution of mammals two great advances were made: firstly, with the exception of the egg-laying monotremes, the embryo now derived protection and nutrition from the mother via a placental connection Secondly, after birth, further nutrition was provided by the mother in the form of milk, thus freeing the newborn from the demands of food-seeking, and allowing it to benefit from a prolonged period of learning under sheltered conditions. In marsupials, of which more later, attachment to a nipple in the mother's pouch occurs at such an early stage that the development that is completed there is more akin to the fetal stages in eutherians. It is interesting to note that evolution of maternal nourishment of the developing embryo is not unique to mammals; some reptiles and snakes have yolk sac placentas, or more rarely allantoic placentas, or in some cases both. There are also some elasmobranchs in which the embryonic yolk sac attaches to the uterine wall and derives nourishment from it (Amoroso, 1952). However, only in mammals is placentation almost universal.

Convenient though viviparity is for the organism concerned, it poses considerable problems for the scientist who wishes to

Abbreviations used in this paper: de, decidual extract; ee, embryonic extract; IC, immediately centrifuged; MEM, Minimal Essential Medium (Eagle's); DMEM, Dulbecco's Modification of Eagle's Medium.

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Printed in Spain

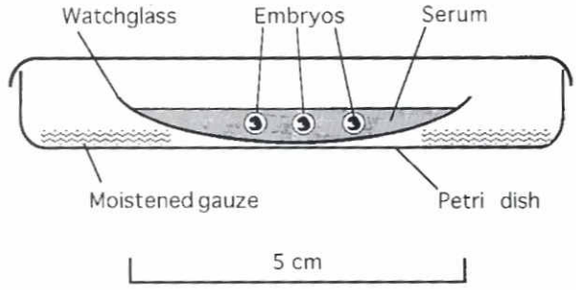

Fig. 1. Watchglass culture apparatus: *several of these can be stacked in a gas-tight chamber within the incubator.*

understand mammalian development. Some *in vivo* techniques are applicable: conceptuses can be injected *in utero* if the precise target is not too critical (Weissman *et al.*, 1978; Papaioannou, 1990), and in mice, embryos at 11.5 days and beyond can be exteriorized from the uterus *('exo utero')* with the placental attachment maintained (Muneoka *et al.*, 1990). These techniques have proved valuable for certain types of study, but in many cases explantation into culture is the preferred technique. In this brief review of culture methods, I shall be concerned mainly with postimplantation mammals, with only a brief mention of other vertebrates. This is not to diminish in any way the latter, but postimplantation mammalian development is the field I know best, and the one that Denis New, whom we honour with this volume, has made his own during the past 35 years.

Chick embryo cultures

Although best known amongst mammalian embryologists for his work on postimplantation mammalian embryos in culture, Denis New is also well known in the chick fraternity, as the creator of the eponymous New-ring technique. From the time of laying, chick embryos have all the materials they need for development within the egg, with the exception of oxygen. However, the hard shell and mass of yolk are impediments to the embryologist. Whilst superficial observations and surgical procedures may be done by windowing (i.e. making a hole in the shell) or emptying the entire egg contents into a bowl, more precise operations and observations are dependent on separating the embryo from the yolk. One such early technique, derived from organ culture, involved explantation of the blastoderm onto a clot consisting of fowl plasma and embryo extract (Waddington, 1932). Valuable though this method was, expansion of the blastoderm over the clot surface did not take place, and embryos rarely developed a blood circulation. In 1955, Denis New published an improved method, essentially supporting the blastoderm on a piece of vitelline membrane stretched beneath a glass ring in a watchglass, nutrition being provided by a little egg albumen beneath the explant (New, 1955). Under such conditions the blastoderm expands normally, and well developed embryos with a functional blood circulation are frequently obtained. The article by Stern and Bachvarova in this volume compares development using this, now classic, technique with a number of modifications and alternatives, and concludes that the original New-ring method stands up well in the company of subsequent innovations. Of course, there are further problems in studying avian development prior to laying, though these stages are amenable to explantation with their yolk, and indeed such preparations can be continued to hatching (Perry, 1988).

Preimplantation mammalian cultures

Culture techniques for mammalian embryos can conveniently be divided into pre- and post-implantation methods. Prior to implantation, the unattached embryos can be flushed out of the oviducts or uterus, and either transferred immediately to another animal, or cultured in relatively simple media (which may be followed by transfer). Below are some of the landmarks in experimentation with preimplantation mammalian embryos.

Success with embryo transfers preceded that with culture of preimplantation embryos, with the first embryo transfer being attributed to Walter Heape (Heape, 1890) using rabbits. As well as offering unequivocal proof of the viability of embryos manipulated *in vitro*, the technique of embryo transfer greatly expands the scope of the questions that can be asked of, and answered by, experimentation on preimplantation embryos. Chang demonstrated that embryos could remain viable during several days storage at low (~10°C) temperatures (Chang, 1947). Long-term storage of embryos became feasible when techniques for reversibly freezing embryos were perfected (Whittingham *et al.*, 1972; Wilmut, 1972). Subsequently such techniques were applied to other laboratory and agriculturally important animals, and of course are now exploited in the fertility treatment of humans.

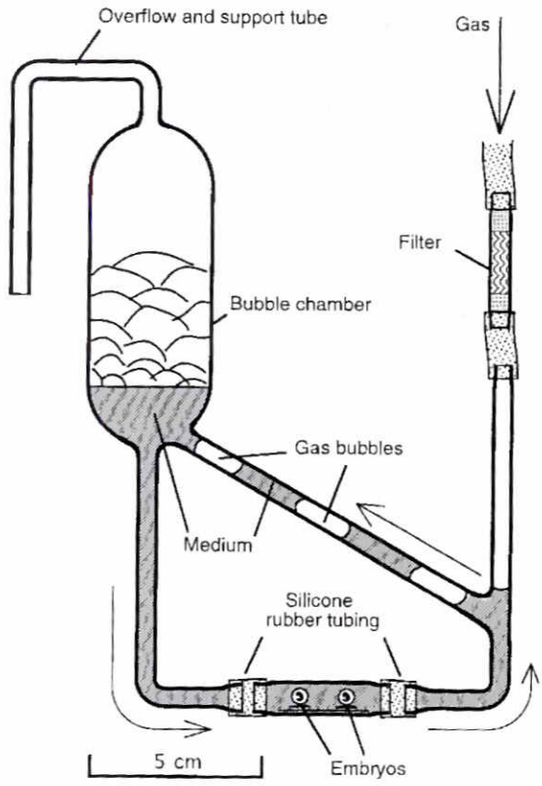

Fig. 2. Circulator culture apparatus. *A device for growing embryos in oxygenated flowing medium. Arrows indicate the direction of gas and medium flow.*

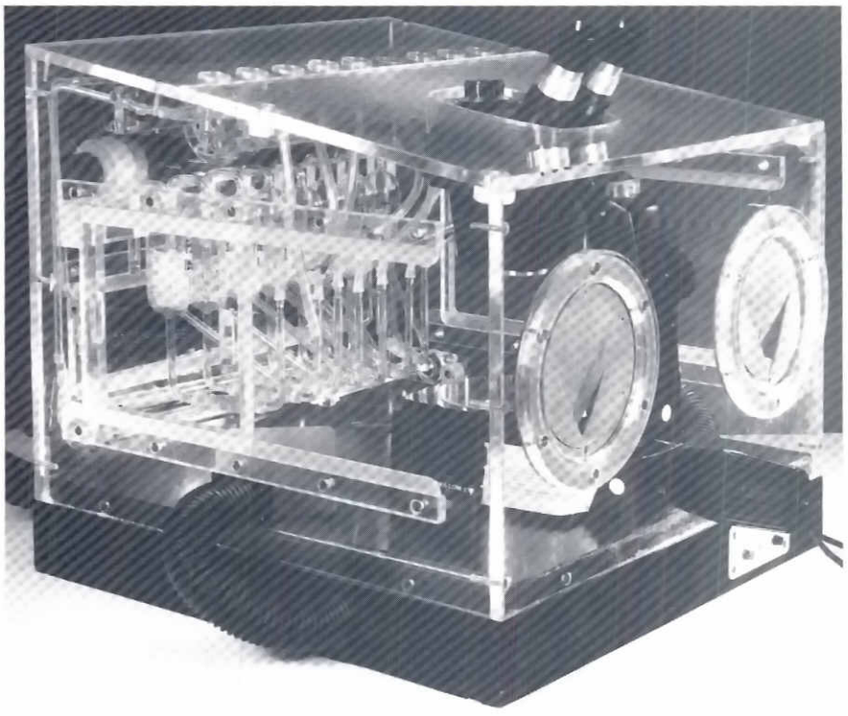

Fig. 3. Incubator for circulator cultures. *Purpose made incubator housing circulators and a binocular microscope.*

Brachet (1912) is generally acknowledged as the first to grow mammalian (5.5-6.5-day rabbit) embryos in culture. In the fifty or so years that followed, preimplantation embryos of a variety of mammals were successfully cultured (see Pincus, 1936; Austin, 1961; Chang, 1981 for reviews). The early cleavage stages in the rabbit were described in some detail by Lewis and Gregory (1929), with the aid of time-lapse cinematography of embryos *in vitro*. Culture of mouse embryos in a relatively complex biological medium, containing yolk and white from hen's eggs, was achieved by Hammond (1949). Culture of mouse embryos in a simpler defined medium, containing bovine serum albumin, was achieved some years later by Whitten (1956). Uterine transfer followed by live birth of mice derived from embryos cultured at preimplantation stages soon followed (McLaren and Biggers, 1958). The above-cited results of Hammond and Whitten were obtained with embryos explanted at the eight-cell stage. Two-cell embryos proved more refractory to culture, though Whitten (1957) found some 40% of such embryos would develop to the blastula (blastocyst) stage with modification of the medium. Relatively large-scale cultures of mouse embryos from the two-cell stage, with 60-100% forming normal blastocysts, were accomplished by Brinster (1963), using a specially formulated medium. Although a number of workers claimed fertilization *in vitro* by cytological criteria, unequivocal proof, by birth of healthy young following fertilization and transfer to prepared recipients, did not come until the work of Chang (1959).

Postimplantation mammalian cultures

Placentation

When implantation takes place a more or less intimate attachment forms between embryo and mother. In marsupials *in utero*, this may be little more than juxtaposition of fetal membranes to the uterine wall, with the young being born at a relatively early stage, and completing their development in the pouch (Tyndale-Biscoe, 1973). In eutherian mammals the nutrition of the embryo is mediated by the (chorio-allantoic) placenta, which allows exchange of materials between maternal and fetal blood circulations, and in its most primitive form comprises six intervening layers, three of maternal and three of fetal origin (the *epithelio-chorial* placenta). This arrangement is still found in, for example, horses and pigs, and in the latter the degree of interdigitation at the maternal/fetal junction is quite modest, and the components are easily separated. In what is arguably the most advanced form of chorio-allantoic placenta, seen in primates and rodents, all three maternal layers break down, so that fetal placental tissues are bathed directly with maternal blood (hence the *hemochorial* placenta). This presumably optimises exchange of materials between the two bloodstreams, whilst maintaining the vital immunological barrier which precludes mixing of maternal and fetal blood.

To study developmental events during the postimplantation phase of development in mammals, it is first necessary to gain access to the

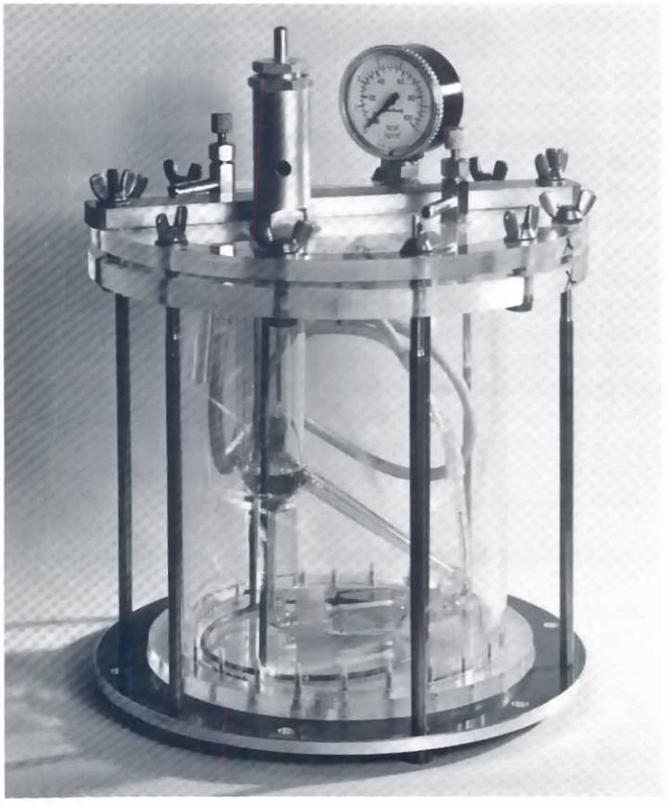

Fig. 4. Pressure chamber apparatus. *Pressure chamber containing circulators that can operate at two or three atmospheres of pressure.*

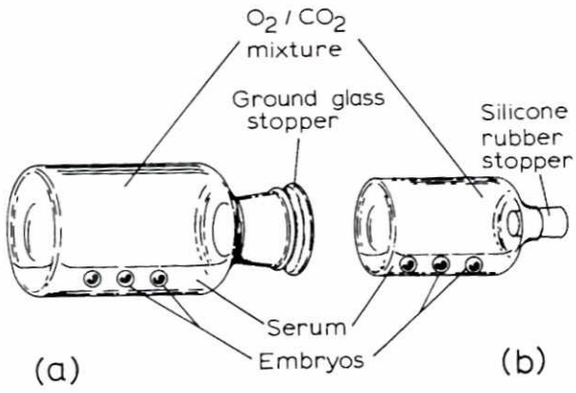

Fig. 5. Two types of roller bottle cultures: *The bottle shown in* **(a)** *is a standard 60 ml reagent bottle sealed with a little silicone grease around the glass stopper, whilst that in* **(b)** *is a purpose made Pyrex bottle, sealed with a silicone rubber bung. (From Cockroft, 1990).*

embryo. Most attempts to do this have involved separating the embryo completely from the mother and incubating it in a culture medium equilibrated with an appropriate gas mixture. Unfortunately for the scientist, attempts to grow a chorio-allantoic placenta *in vitro* have been largely unsuccessful, although it has been demonstrated that an allantoic circulation can be established in culture (New and Coppola, 1977). Fortunately, in rodents at least, there is a transitory period during early organogenesis when the chief nutritive organ is the yolk sac. The inner layer of this, known as the *visceral yolk sac*, becomes inverted and vascularized, so that *in vivo* absorptive endoderm cells are separated from maternal blood only by the thin outer *parietal yolk sac* (containing Reichert's membrane). When embryos have grown beyond the size where simple diffusion in culture can satisfy their needs, the visceral yolk sac provides the necessary support by facilitating nutritional and respiratory exchanges between the embryo and culture medium. The parietal yolk sac is usually opened or removed, although it will undergo some expansion *in vitro* if left intact (Ellington and New, 1980; Cockroft, 1987). Whilst yolk-sac mediated nutrition can maintain the embryo for only a few days at most, the speed of rodent development is such that the undifferentiated embryo can develop in culture to early fetal stages with the formation of rudiments of all the main organs. The contributions to this journal issue demonstrate the variety of knowledge that has been gained from such postimplantation culture techniques.

Evolution of postimplantation culture techniques

Culture methods

During the 1930's a number of workers experimented with culturing the embryos of the rat (Nicholas and Rudnick, 1934, 1938; Goss, 1935; Nicholas, 1938), rabbit (Waddington and Waterman, 1933) and guinea pig (Jolly and Lieure, 1938). Although they succeeded in demonstrating that postimplantation development could be obtained in culture, such methods were not widely adopted until Denis New and his colleagues introduced various improvements in the 1960's and 1970's. It was soon established that culture in all-liquid media (New, 1966) gave better results than culture on plasma clots (New and Stein, 1964) – the classic method for organ culture (Fell and Robison, 1929). A number of Petri dishes, each containing a watchglass with embryos and liquid medium (Fig. 1), were stacked in a rack and covered with an inverted beaker over a larger diameter dish containing sufficient paraffin oil to form a gas-tight seal with the rim of the beaker. The air in the chamber was replaced with the desired gas mixture and the entire apparatus then housed in a 37°C incubator. Using this system, New (1966) obtained good development of 6-12 somite rat and mouse embryos up to limb bud stages with 24-32 somites, and noted that the older, but not the younger, embryos benefited from higher (60%) oxygen concentrations.

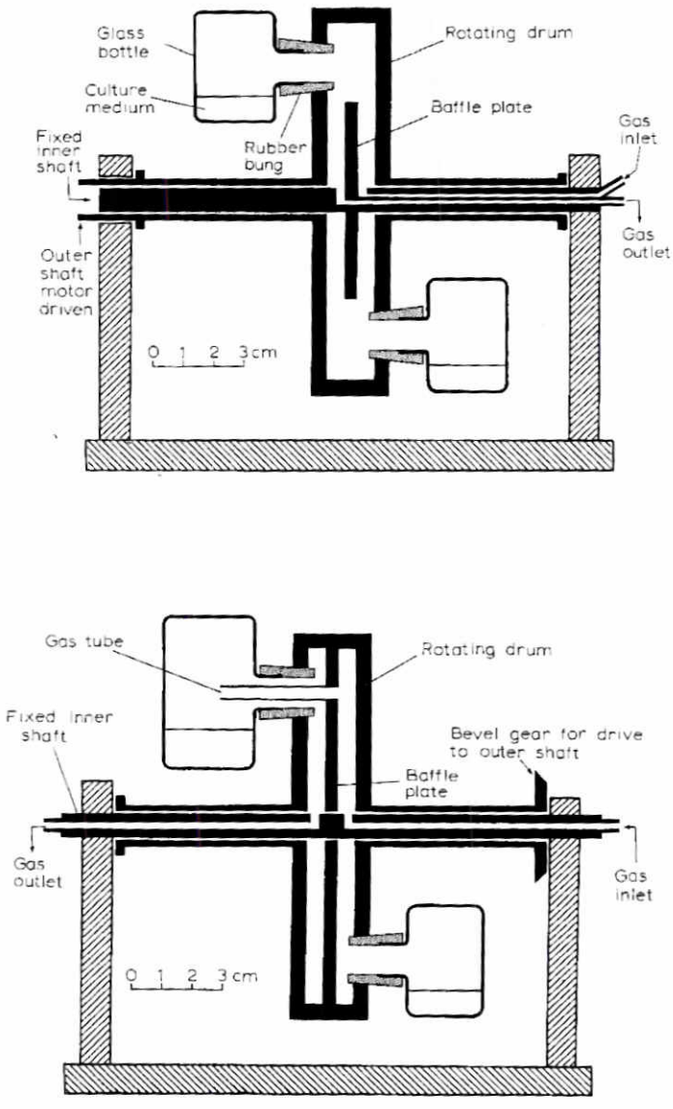

Fig. 6. Cross sections of two types of rotator apparatus. *Two models of rotator apparatus, in which the rotating bottles are constantly gassed by diffusion (upper diagram) or via tubes projecting into the bottles (lower diagram). (From New and Cockroft, 1979).*

TABLE 1

SOME EXAMPLES OF CULTURE MEDIA THAT HAVE BEEN APPLIED TO POSTIMPLANTATION EMBRYOS

Stage at explantation	Somites added during culture	Culture medium	Workers
Rat			
late streak	10 to 12	rat plasma + rat de	Jolly and Lieure, 1938
9 day	?	rat/cat plasma + pig amniotic fluid + Tyrode saline	Nicholas, 1938
9 day (headfold)	6 to 10	rat plasma ee clot	Nicholas and Rudnick, 1938
10.5 day (6 to 12 somites)	~18	chick or rat plasma + rat ee clot	New and Stein, 1964
11 day (25 somites)	5 to 6	human serum	Shepard *et al.*, 1970
12.5 to 13.5 day	8 to 10	25% rat serum + 75% Tyrode saline	Cockroft, 1973
9.5 day (headfold)	35 to 45	100% IC rat serum	New *et al.*, 1976a
7.5 to 8.5 day (egg cylinder)	30 to 35	100% IC rat serum	Buckley *et al.*, 1978
9.5 day (headfold)	~20	monkey serum	Klein *et al.*, 1982
Mouse			
8 to 9 day (8 to 12 somites)	~18	chick plasma + chick/mouse ee clot	New and Stein, 1964
8.5 day (4 to 7 somites)	~ 8	horse serum + Hanks saline + chick ee + agar	Smith, 1964
7.75 day (headfold)	?	Eagles MEM + 9% rat serum	Tamarin and Jones, 1968
1 to 12 somites	~12	Waymouth's 752/1+ 20% rat serum	Clarkson *et al.*, 1969
0 to 4 somites	'numerous'	Eagles BME + 10% FCS + 2.5% chick ee	Moore and Metcalf, 1970
10.5 day (30 somites)	12	50% FCS, 50% Waymouth's	Kochhar, 1975
8.5 day (2 to 4 somites)	~25	100% IC rat serum	Sadler, 1979
7.5 day (headfold)	23	100% IC rat serum	Sadler and New, 1981
7.5 day (late streak)	20	50% IC rat serum, 50% DMEM	Lawson *et al.*, 1986
6.7 day (pre-early streak)	0 to 8	50% IC rat serum, 50% DMEM	Lawson *et al.*, 1987
Rabbit			
late streak	~6	chick plasma + chick/rat ee clot	Waddington and Waterman, 1933
6.75 day (pre-streak)	5 to 20	F10 + 20-25% rabbit serum	Daniel, 1970
Guinea pig			
late streak	10 to 12	guinea pig plasma + guinea pig de	Jolly and Lieure, 1938
Hamster			
8 day	?	McCoys 5A + 30% hamster serum	Givelber and DiPaolo, 1968
Opossum			
7.5 to 12 day	3 to 7	199 + 20% opossum serum	New *et al.*, 1977

Abbreviations: de, decidual extract; ee, embryonic extract; IC, immediately centrifuged.

In these watchglass cultures, it seemed likely that a limiting factor was the slow diffusion rate of nutrients and respiratory gases through the static culture medium. A method was required for maintaining a flow of continuously oxygenated medium past the embryo. After a number of trials, an apparatus was devised (New, 1967) that was simple and effective and came to be known as a 'circulator' (Fig. 2). Gas bubbling up the sloping arm of a triangle of glass tubing both oxygenated the medium and carried it round the triangular circuit, past the embryos which were anchored to a collagen-coated 'raft'. This system gave significantly improved development, particularly of the older embryos. The incubator containing the circulators also housed a binocular microscope, so that embryos could be observed, intermittently or continuously, without cooling or interruption of the gas flow (Fig. 3). A notable early application of the circulator was in the laboratory of Tom Shepard in Seattle. In collaboration with Tanimura and Robkin, Shepard performed studies on the energy metabolism of mid-gestation rat embryos that remain to this day amongst the most elegant and significant applications of postimplantation culture techniques (Shepard *et al.*, 1970). In these studies a further feature of the circulator was exploited: it is possible to analyze the gas perfusing the embryos, in this instance by absorbing radiolabeled carbon dioxide with potassium hydroxide solution which was subsequently analyzed as part of the process of assessing the level of activity of oxidative energy pathways in the embryos.

Although New's circulator was simpler than other methods of circulating the medium, which generally required mechanical pumps and a separate oxygenating system (e.g. Givelber and DiPaolo, 1968; Tamarin and Jones, 1968; Robkin *et al.*, 1972), it was not widely adopted. The apparatus was specialized and not all laboratories had access to the necessary glassblowing and engineering services. Furthermore, setting up the cultures was time consuming, as was cleaning the circulators by immersion in chromic acid followed by copious rinses. Nevertheless, the system was pushed to its limits, most memorably in attempts to culture more advanced embryos by housing the circulators in a pressure chamber (Fig. 4) and operating them in two or three atmospheres of almost pure oxygen (New and Coppola, 1970a). This did indeed produce somewhat larger and more advanced embryos, but the high oxygen levels soon damaged and impaired the function of the yolk sac, curtailing any further embryonic development (New and Coppola, 1970b), and the additional complications introduced by the pressure chamber put a limit on the general applicability of the system. As it turned out, a more effective and safer method of increasing the oxygen supply and prolonging development was to open the yolk sac and exteriorize the embryos (Cockroft, 1973), allowing nutrient and gaseous interchange through the fetal skin (see below).

But it was a method for culturing in rotating bottles (New *et al.*, 1973) that was eventually adopted in laboratories world-wide. In this method, a small part of the volume of each bottle is occupied by the embryos and culture medium, and the remainder by a suitable gas mixture (Fig. 5). The bottles are sealed and continuously rotated on rollers within the incubator. The rotation-induced swirling of the culture medium facilitates gaseous and nutrient interchange between embryo and medium, and also oxygenation of the medium by the gas phase. The simple apparatus – any bottle or tube (including disposable plastic tubes) that can be made gas-

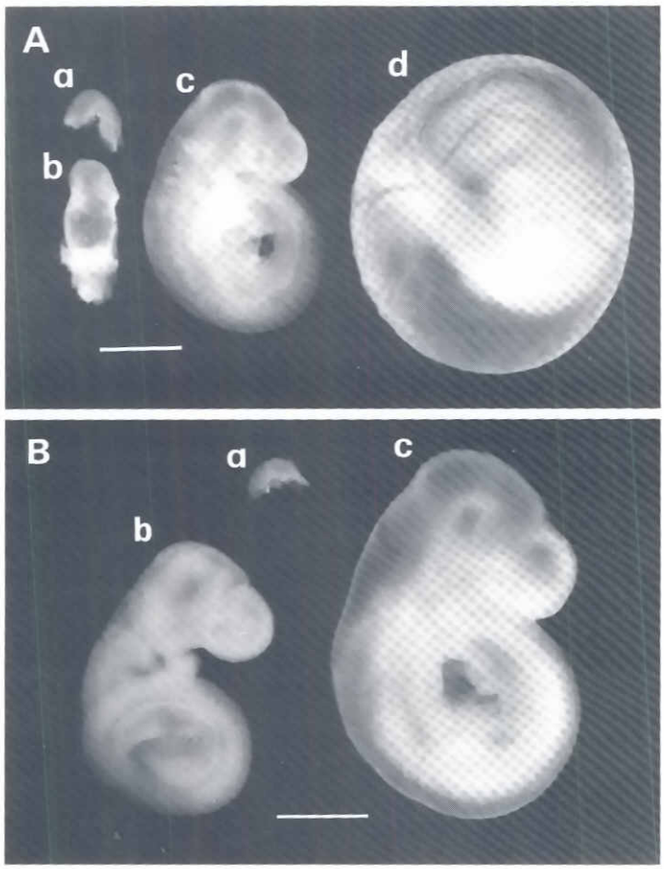

Fig. 7. Culture of 9.5-day rat embryos. (A) *Rat embryos explanted at 9.5 days, before (a,b) and after (c,d) a 48 h culture. Embryos are shown dissected free of their extraembryonic membranes (a,c), and with the membranes intact (b,d), as during culture.* **(B)** *With renewal of the medium after 48 h (b), culture of rat embryos from the head-fold stage (a) can be continued to 72 h (c) (shown free of the membranes with which they were cultured). Bars, 1 mm.*

TABLE 2

POPULAR CULTURE REGIMES FOR RAT AND MOUSE EMBRYOS

Age at explantation (days)	Initial somites	Culture period (h)	Final somites
Mouse			
7.5 (pm)	0 (headfold)	36-48	15-23
8.5 (am)	2-5	36-48	22-28
9.5 (pm)	20-24	18-24	26-32
Rat			
8.5 (pm)	0 (primitive streak)	60-65	~25
9.5 (am)	0 (head-fold)	48-72	26-35
10.5 (am)	10-15	45	35-40
11.5 (pm)	26-28	18-24	~40
12.5 (pm)	40-44 (open yolk sac)	42	50-55
13.5 (pm)	51-55 (open yolk sac)	42	60-63

tight and is non-toxic will suffice – together with the simpler explantation procedure means that large numbers of embryos can be cultured without undue labour or expense. As an alternative to placing the bottles on rollers, a method was also devised for attaching them (unstoppered) to a rotating hollow drum (New and Cockroft, 1979). An appropriate gas mixture, fed continuously to the drum along its hollow axle, constantly refreshes the atmosphere within the bottles, either by diffusion or via tubes projecting into each bottle (Fig. 6). The result is improved maintenance of oxygen and carbon dioxide/pH levels without any laborious gassing and regassing of bottles individually. Hence the simplicity of the rotating bottle is allied to the better maintenance of pH and gas composition that is characteristic of the circulator. Neither of the two rotating bottle methods allows for the continuous observation of the embryos that is possible with the circulator, but this is usually more than compensated for by their greater convenience and versatility.

Culture medium

Along with the introduction of the rotating bottle systems, improvements were made in the culture medium. Table 1 gives some idea of the variety of media that have been applied to postimplantation embryos in culture. Early tests had shown that, for rat embryos, homologous serum was best (New, 1966). When this was prepared by the standard technique, with the blood allowed to clot at 4°C overnight followed by centrifugation, it was satisfactory for somite-stage embryos, but head-fold and earlier stages were frequently abnormal. Chris Steele, whilst a PhD student in Denis New's laboratory, discovered that preparing the serum from blood centrifuged immediately after withdrawal from

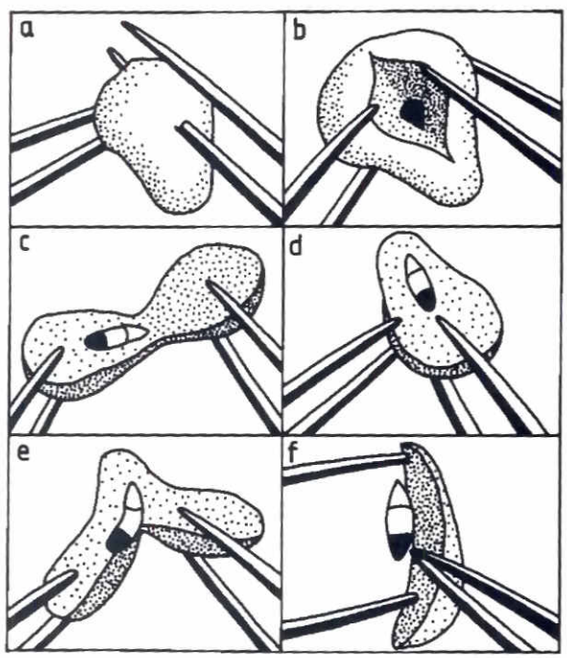

Fig. 8. Dissection procedure for 9.5-day rat embryo. *Sequence of operations in removal of the decidua (stippled) from the 9.5-day (early head-fold) rat conceptus, using watchmaker's forceps. Explantation is completed by reflection of the parietal yolk sac. (From Cockroft, 1990).*

the donor prevented the formation of double hearts in embryos developing from primitive-streak stages (Steele, 1972; Steele and New, 1974), and it was later shown that such serum, often abbreviated as I.C. serum, also gave more reliable development of head-fold stages (New *et al.*, 1976a). For both practical and scientific reasons, a fully defined culture medium would be invaluable. However, although there has been some success in defining the micromolecular components of the culture medium (Gunberg, 1976; Cockroft, 1979, 1988), there remains some essential factor(s) with an apparent molecular weight in excess of 12,000 daltons which as yet can only be provided by serum (usually from rats). Some proteins have been identified that are depleted in serum used for embryo culture (Klein *et al.*, 1978; Priscott *et al.*, 1983), but still the magic ingredient(s) remain elusive.

Oxygen

Maintenance of the optimum oxygen level is also critical to the success of cultures. Early somite stage embryos develop well in culture medium equilibrated with air (with 5% carbon dioxide). Older embryos require higher oxygen concentrations, largely to compensate for the lack of an adequate placental transport system. But a more surprising discovery was that presomite embryos require *lower* oxygen levels than that obtained by equilibration with air (i.e. less than 20% oxygen), and for them 5% oxygen was found to be beneficial (New *et al.*, 1976a). The culture procedure that has been most widely adopted and adapted is to incubate head-fold or early somite stage rat (or mouse) embryos for two days in rotating bottles, in immediately centrifuged rat serum equilibrated initially with 5% oxygen, raised to 20% oxygen around the time that a heart-beat and blood circulation develop (Fig. 7). It has been demonstrated that this regime gives development barely distinguishable from *in vivo* (New *et al.*, 1976b) and since this procedure was established, there has been little substantive improvement in method, but a burgeoning of applications of the technique in diverse developmental fields. Cultures of the head-fold rat embryo can reliably be continued for 72 h (Fig. 7) if the culture serum is renewed, but often the shorter period is sufficient to show the effect under study, and therefore more economical.

Although the culture methods were largely developed using rat embryos, which are somewhat easier to work with, particularly at early stages, similar results are also attainable with the mouse (Sadler, 1979; Sadler and New, 1981; Lawson *et al.*, 1986, 1987; Martin and Cockroft, 1997). Some popular culture protocols for the rat and mouse are shown in Table 2.

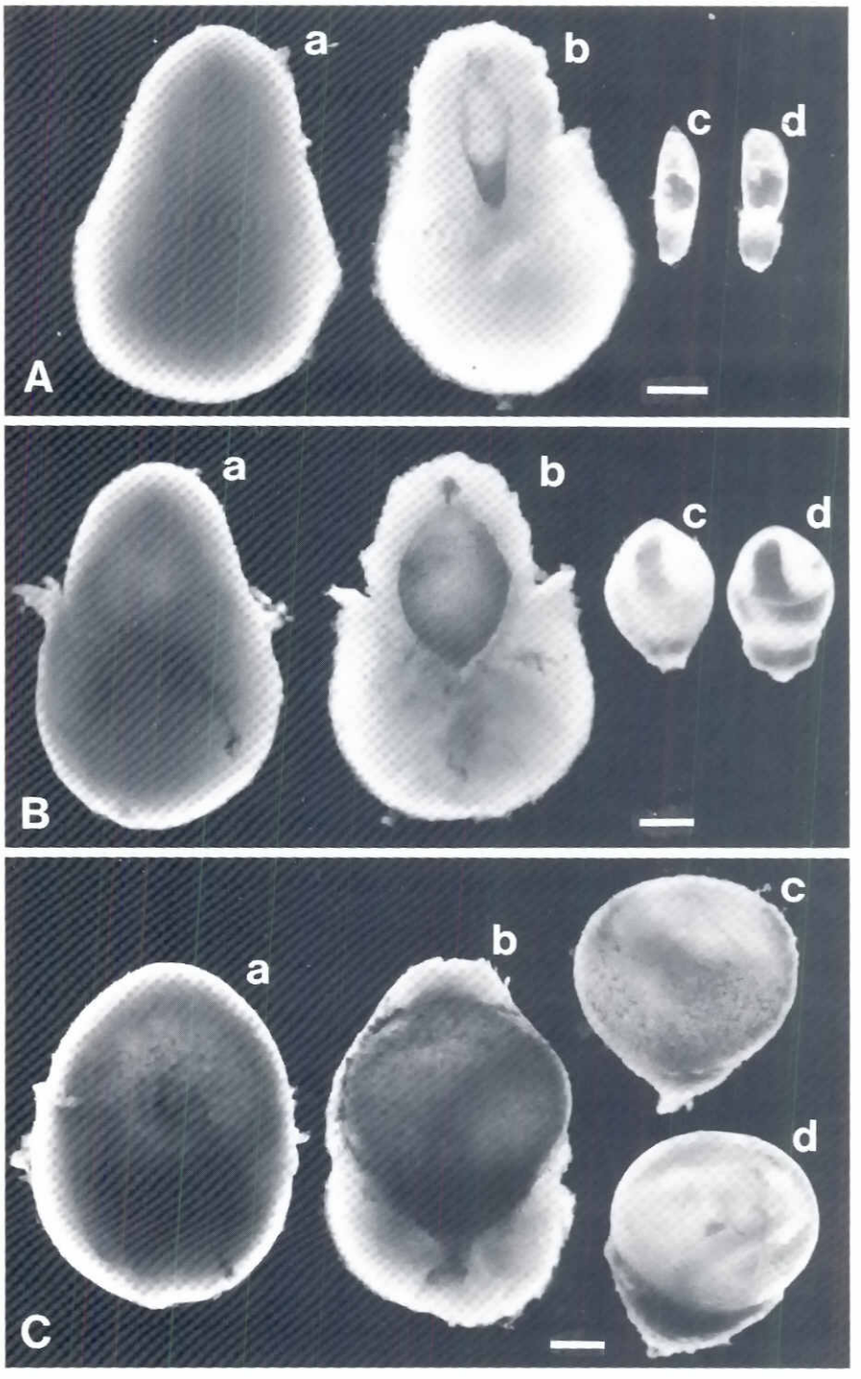

Fig. 9. Dissection of 9.5-, 10.5- and 11.5-day rat embryos. *Stages in the dissection of* **(A)** *9.5-day,* **(B)** *10.5-day, and* **(C)** *11.5-day rat embryos. In each case: (a) On removal from the uterus, the conceptus is embedded in maternal decidua; (b) half the decidua has been removed, showing the position of the conceptus within; (c) the decidua has been completely removed; (d) with the parietal yolk sac opened, the conceptus is ready for culture. Bars, 1 mm.*

Dissection techniques

For most of the stages that have been successfully cultured, the dissection procedures are fundamentally similar (Figs. 8 and 9): the conceptus is separated from the uterus, the decidua in which it is embedded is peeled off, and the parietal yolk sac

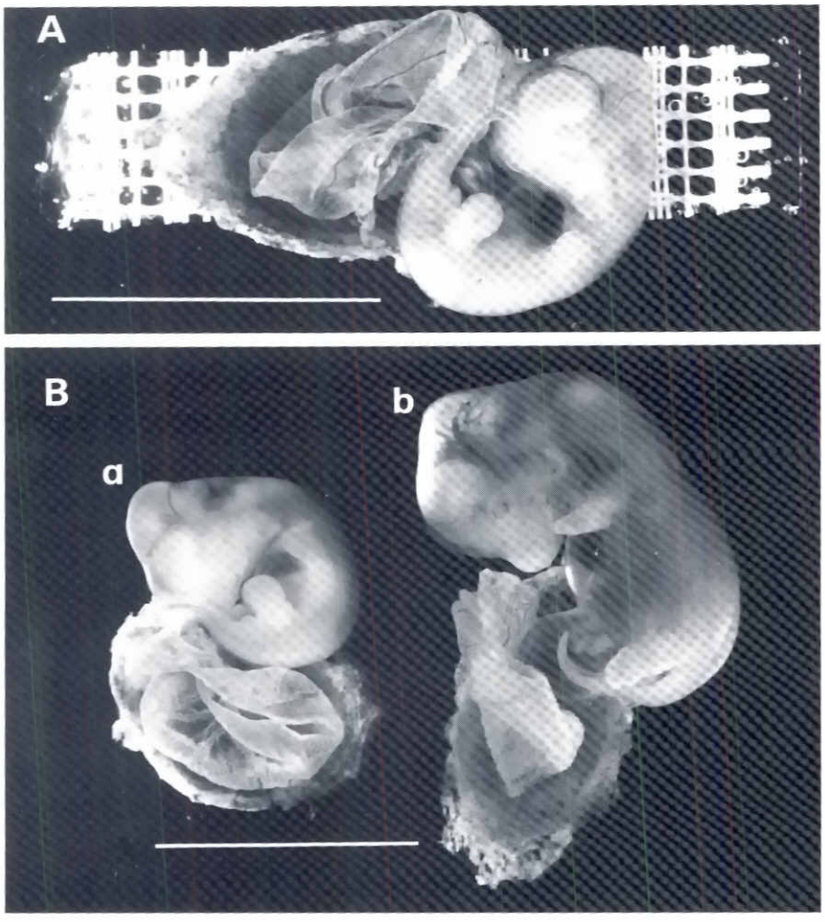

Fig. 10. Culture of 13.5-day rat embryos. (A) *Rat embryo explanted at 13.5 days with open (visceral) yolk sac is stuck to a fabric-covered, collagen-coated, glass coverslip, for anchorage within a circulator (see Figs. 2 and 3).* **(B)** *Rat embryos explanted at 13.5 days before (a) and after (b) 42 h of culture with open yolk sacs. Bars, 10 mm.*

(Reichert's membrane with its adherent trophoblast and parietal endoderm cells) is opened, exposing the visceral yolk sac endoderm layer (see New, 1971; Cockroft, 1990). However, there are some variations for older and younger embryos that are worthy of mention. One of the most difficult stages to culture successfully is the primitive-streak (or pre-streak) stage, (8.5 days in the rat, 7.5 days in the mouse); so far, even the most successful cultures have shown a 20% malformation rate (e.g. Buckley *et al.*, 1978). One study appeared to improve on this, modifying the explantation procedure by transecting the extraembryonic visceral yolk sac and thus removing the ectoplacental cone (Fujinaga and Baden, 1991). Embryos explanted by this procedure did indeed develop well, with 100% (41/41) of 8.5-day rat embryos developing normally to the 26-somite stage. But equally noteworthy was that in their study, the control embryos explanted by the 'old' technique also developed very well to the same stage with only 7.3% (3/41) developing abnormally. Thus it appears that some factor other than removal of the ectoplacental cone may have been responsible for the good development of primitive streak rat embryos in this study. Notwithstanding, the authors had fewer embryos damaged (and therefore discarded) during dissection by their novel technique, and found the method easier, which would recommend the technique to those with the same experience.

The second variation in dissection technique, which has already been alluded to, improves the culture of the most advanced embryos – rat embryos explanted at 12.5-13.5 days (Cockroft, 1973, 1976); mouse embryos at 11.5-12.5 days (Martin and Cockroft, 1997) – by opening the yolk sac and thereby exposing the fetal surface to the culture medium (Fig. 10). Using 95% oxygen at one atmosphere, this procedure improves the development in culture of these stages, presumably by increasing the available surface for gaseous and/or nutrient interchange. Although originally used with circulator cultures, this dissection method also works with rotating bottle cultures (Martin and Cockroft, 1997).

Marsupials

Unfortunately there is no true equivalent with mammalian postimplantation culture of the embryo-transfer technique that has proved so valuable in the study of the preimplantation mammalian embryo. A notable attempt was made by injection of 6th and 7th day mouse embryos into the decidua of 5th or 6th day pregnant mice, using pigmentation and enzyme markers to distinguish host and donor conceptuses (Beddington, 1985). Whilst a small proportion of such embryos reimplanted and developed into 12th-14th day fetuses, none went to term, possibly due to retarded development of the allantoic placenta. Denis New had previously brought a different approach to this problem, reasoning that if the present techniques could carry development only to the limb-bud stage, it would be interesting to examine the possibility of culturing embryos of a species that is actually born at about this stage, i.e. a marsupial. To this end some trials were made with the Virginian opossum in collaboration with Merle Mizell (New and Mizell, 1971; New *et al.*, 1977), and some development in culture of opossums at various stages was achieved, including with embryos close to birth (Fig. 11). The practical difficulties of working in quarantine with such a large and reproductively intransigent animal meant that the goal of transferring cultured embryos to the pouch for continued development was not attained. Nevertheless, marsupials remain an attractive subject for culture studies – see, for example, the contribution by Selwood *et al.* to this volume.

Future challenges

The articles in this volume represent only a fraction of the applications which have benefited from vertebrate culture techniques. Whilst acknowledging all that has been achieved, many challenges remain. Those that come most immediately to my mind are:

a) Improving the culture of early postimplantation stages (primitive and pre-primitive streak).
b) Obtaining culture over the implantation period; some remarkable success in this area has been reported by Hsu and his

colleagues – e.g. Hsu (1972, 1979), Hsu *et al.* (1974) – but have not yet been repeated with the same level of success elsewhere.

c) Extending the upper limit of culture, perhaps using an artificial placenta or cannulation of the umbilical vessels.
d) Continuing the development of cultured embryos to term, by reimplantation or other means.
e) Formulation of a fully defined culture medium for those stages that are amenable to culture.

Whatever the future holds for vertebrate development *in vitro*, it will be on the solid foundations laid by Denis New.

Summary

In the culture of chick embryos, one of the most significant advances has been the introduction of the New-ring technique (after Denis New, its originator). For preimplantation mammalian embryos, techniques developed over the past century allow manipulation and culture throughout the preimplantation phase, indefinite storage when frozen, and birth of healthy offspring following transfer of cultured embryos to prepared recipients. Many of the advances in post-implantation embryo culture were devised in the 1960's and 1970's by Denis New and his colleagues. It was found that culture in flowing or swirling medium gives better development than with static cultures, and this is most simply achieved in rotating bottle cultures. Culture in liquid medium gives results superior to those obtained on plasma/embryonic extract clots, and if the medium is serum centrifuged immediately on withdrawal from donor rats (before clotting), the frequency of embryonic abnormalities is reduced. Embryos at primitive-streak or head-fold stages benefit from a lower oxygen concentration (5%) than is achieved by equilibrating the culture medium with air (i.e. 20% oxygen). At around the time that the heart-beat and blood circulation are established (10-15 somites), the latter concentration becomes optimal, and by the 25-30 somite stage, 95% oxygen is needed. Development of embryos beyond 40-45 somites is best achieved by opening the visceral yolk sac so that gaseous and/or nutrient exchange can occur via the fetal skin.

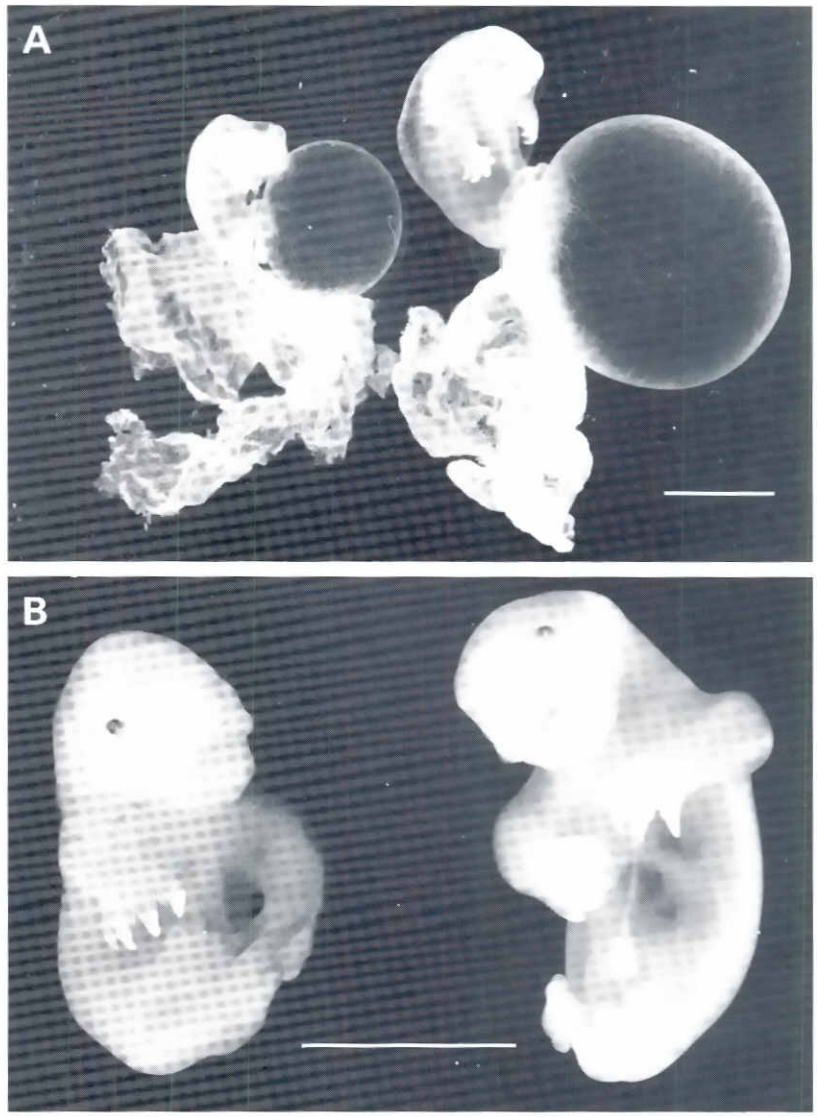

Fig. 11. Cultures of opossum embryos. *Opossum embryos at* **(A)** *11.5 days, before (left) and after (right) 26h of culture, and* **(B)** *12.0 days, before (left) and after (right) 11h of culture. Bar, 5 mm.*

KEY WORDS: *culture, mammalian, postimplantation, preimplantation, chick*

Acknowledgments

I thank Professor Richard Gardner and A.N.Other for constructive criticism of the manuscript, Mrs. Ann Yates and Ms. Rebecca Stack for assistance with its preparation, and the Imperial Cancer Research Fund for financial support.

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