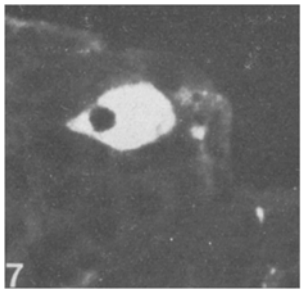
omission du premier temps de la réaction, utilisation d'un simple sérum de lapin ou enfin, application de l'Ac. préalablement saturé par l'antigène correspondant.

Résultats. Parmi tous les Ac. utilisés, seul l'Ac. anti-α endorphine nous a permis d'observer une immunofluorescence positive au niveau de 4 cellules des ganglions infra-œsophagiens: a) 2 cellules coniques très antérieures, symétriques et situées au point de pénétration des connectifs périœsophagiens (30 µm × 20 µm, figures 1–3). b) 2 cellules piriformes postérieures, latérodorsales, symétriques et situées en avant du dernier nerf ganglionnaire (40 µm × 25 µm, figures 4–6). La partie proximale de leur axone, très fine, est parfois visible en fluorescence sur une longueur de 20 µm environ. Le nombre, la position de ces cellules, ainsi que leur affinité pour l'Ac. anti-α-endorphine ont été rigoureusement identiques chez tous les animaux examinés. La taille relativement importante des péricaryons nous a permis, sur des coupes contiguës de la même cellule, de comparer les réactions avec les différents Ac. ou de s'assurer de la spécificité de cette réaction, et notamment qu'un Ac. anti-α-endorphine saturé par α-endorphine ne provoque aucune fluorescence (figures 7 et 8). En montant des coupes voisines sur 2 lames distinctes, nous avons réalisé différentes colorations classiques. Les 4 cellules décrites ne présentent aucune affinité tinctoriale remarquable: elles sont fuchsine-paraldéhyde et hématoxyline chromique négatives, elles se colorent en violet avec l'azan comme de nombreuses autres cellules de ces ganglions. Ces cellules ne semblent donc correspondre à aucune des cellules neurosécrétrices décrites à ce jour dans le système nerveux central de *Dendrobaena* ou d'autres lombricidés[5-7]. Les nombreux sites fuchsine paraldéhyde positifs des ganglions cérébroïdes et infra-œsophagiens de *Dendrobaena* ne présentent aucune réaction croisée avec des Ac. anti-neurophysine et anti-vasopressine, contrairement à ce que nous avons pu observer chez certains insectes[8].

Un neuropeptide comparable à l'α-endorphine est donc présent dans un nombre très limité de cellules du système nerveux central de certaines annélides. Il est intéressant de noter que, comme chez les lépidoptères, ces cellules sont localisées dans les ganglions sous-œsophagiens et absentes des ganglions cérébroïdes. Des études physiologiques doivent permettre de déterminer le rôle de ce neuropeptide chez les invertébrés.



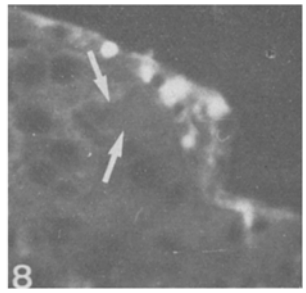


Fig. 7. Ganglions infra-œsophagiens. Coupe transversale traitée par l'Ac. anti-α-endorphine. Fluorescence très nette au niveau de la cellule postérieure droite. × 450.

Fig. 8. Coupe voisine (7 µm) de celle de la figure 7 traitée par l'Ac. anti-α-endorphine saturé par α-endorphine. Absence de fluorescence au niveau de la cellule postérieure droite. × 450.

NB. La fluorescence observée au niveau des bordures est une fluorescence non-spécifique.

1 E.A. Zimmermann, A. Liotta et D.T. Krieger, Cell Tissue Res. *186*, 393 (1978).
2 E. Follénius et M.P. Dubois, C. r. Acad. Sci. *285*, 1065 (1977).
3 Ch. Rémy, J. Girardie et M.P. Dubois, C. r. Acad. Sci. *286*, 651 (1978).
4 M. Goldman, dans: Fluorescent antibody methods, p. 157, Academic Press, New York 1978.
5 J. Michon et F. Alaphilippe, C. r. Acad. Sci. *249*, 835 (1959).
6 H. Herlant-Meewis et N. Van Damme, C. r. Acad. Sci. *255*, 2291 (1962).
7 H. Herlant-Meewis, Z. Zellforsch. *69*, 319 (1966).
8 Ch. Rémy, J. Girardie et M.P. Dubois, C. r. Acad. Sci. *285*, 1495 (1977).

PRO LABORATORIO

A rotating bottle culture method with continuous replacement of the gas phase

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Summary. A 'rotator' culture method is described which provides a continuous flow of oxygenating gas to cultures in rotating bottles. The system maintains constant O_2 and CO_2 levels in the culture medium throughout the incubation period. It also provides a more stable pH than systems with sealed culture bottles.

Satisfactory growth of tissue in culture requires that all the constituent cells can obtain an adequate supply of oxygen and nutrients and can lose carbon dioxide and waste products. When the tissue is very thin, as in an epithelium or cell monolayer, these requirements can be met by diffusion through a static (non-flowing) culture medium and gas phase, as in the standard petri dish type of culture. But growth of tissues more than about 1–2 mm thick, or of bulk cell suspensions, usually requires a continuously flowing medium. Many systems have been devised for circulating and oxygenating the medium[2-4]. Among the simplest and most widely used are methods for growing the tissues in sealed rotating bottles[2,5,6]. Usually about $\frac{1}{5}$ or less of each bottle is filled with the medium and explants, and the remainder with the required $O_2/N_2/CO_2$ gas mixture. The rotation of the bottle promotes oxygenation of the medium by continuously exposing a fresh layer to the gas phase, and maintains a flow of medium over all surfaces of the tissue.

The rotating bottle methods have often given excellent results[7-10]. However, they have the disadvantage that the oxygen and carbon dioxide levels in each bottle may alter during the culture as a result of the respiration of the cultured tissue[10], and may vary from one bottle to another. Although such changes can be minimized by enclosing only

a small amount of tissue and medium in each bottle relative to the volume of the gas phase, and by re-gassing the bottles at intervals during the culture, they cannot be eliminated altogether and may be critical in certain types of experiment.

To overcome this problem we have devised a rotating-bottle system (referred to in this paper as a 'rotator') which allows continuous renewal of the gas phase. The bottles, which remain open during the culture, are attached to a hollow rotating drum (figure 1) through which the required gas mixture is continuously circulated. The method has recently been used to support prolonged development (to early fetal stages) of pre-primitive streak rat embryos[11] which had previously proved very difficult to grow in culture. As there would appear to be many other possible applications, we give here details of the system.

The rotator system. Figure 2 shows the basic design of the rotator, drawn to scale. The hollow drum and outer shaft revolve at about 30 rev/min round the fixed inner shaft. One end of the rotating shaft projects through the side of the incubator (not shown in the diagram) and is driven externally by an electric motor. The oxygenating gas mixture (warmed, filtered and humidified) enters through a longitudinal duct in the inner shaft, circulates round the cavity of the drum and escapes through a 2nd duct in the inner shaft. (To provide these ducts, the inner shaft can conveniently be made from 2 concentric tubes with a space between them). A gas flow rate of a few ml per min is adequate. A circular baffle plate, fixed to the inner shaft, promotes an even circulation of the gas throughout the cavity of the drum. Loss of gas between the inner and outer shafts is prevented with silicone vacuum grease. The entire rotator can be made from aluminium and can be sterilized in a dry oven, without dismantling.

The glass culture bottles are inserted into holes on each face of the rotator drum and held in place by silicone rubber bungs. Bottles of the size shown in figure 2 (35 mm diameter × 30 mm depth, with neck 12 mm diameter, all internal dimensions) each hold up to 6 ml of medium; equilibration of the medium with the gas in the drum is attained in 20–30 min. 12 such bottles can be accommodated on a drum 13 cm in diameter, but any smaller number of bottles may be used provided any unoccupied holes on the drum are closed with bungs.

If much larger bottles are used, or if oxygen consumption is exceptionally rapid, it may be necessary to duct the gas flow into each bottle. A modification of the rotator which provides this is shown in figure 3. The baffle plate here extends across the whole diameter of the drum, completely separating the cavity of the drum into 2 parts. The plate is attached to the circumference of the drum and rotates with it round the fixed inner shaft. Each of the bottles on 1 face of the drum is supplied with gas by a tube passing through the baffle plate and extending into the cavity of the bottle. (The other face of the drum may be used to accomodate bottles without gas tubes).

Oxygen and carbon dioxide content, pH and osmolarity, of the culture serum after 24 h incubation in rotator bottles with a continuous gas supply, and in sealed roller bottles gassed only initially. All the bottles were of identical size and shape and contained 5 ml serum. Bottles 1–4 were without embryos: bottles 5–8 contained 5 embryos each

Bottle	pO_2	pCO_2	pH	Osmolarity
1 rotator	138	37	7.47	295
2 rotator	135	35	7.48	295
3 roller	144	32	7.52	300
4 roller	151	31	7.54	300
5 rotator + embryos	134	37	7.19	294
6 rotator + embryos	133	36	7.22	295
7 roller + embryos	114	73	6.92	300
8 roller + embryos	125	68	6.96	302

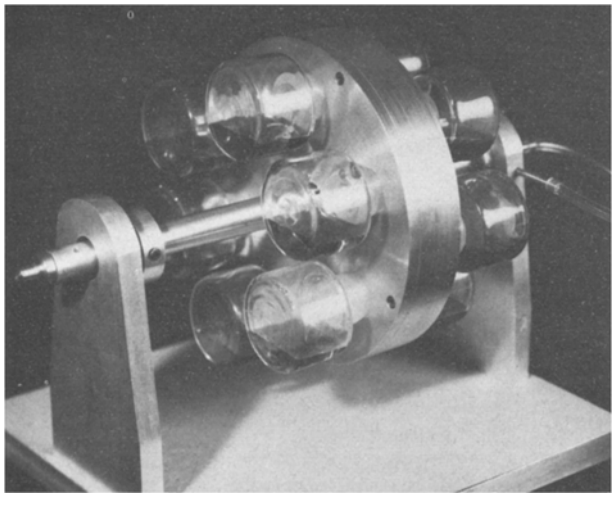

Fig. 1. Rotator with 12 culture bottles and leads for gas flow.

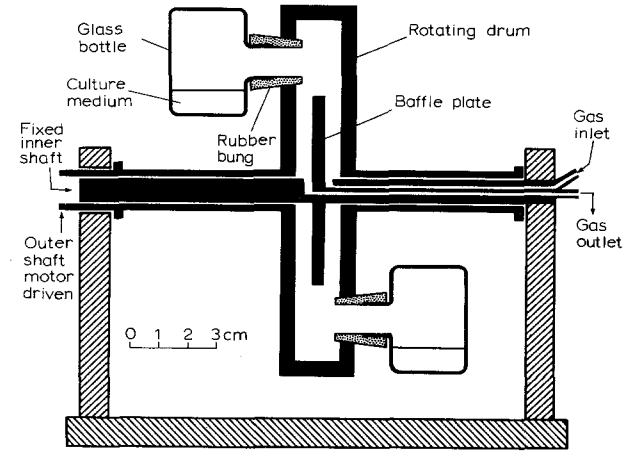

Fig. 2. Diagram showing construction of the rotator of figure 1.

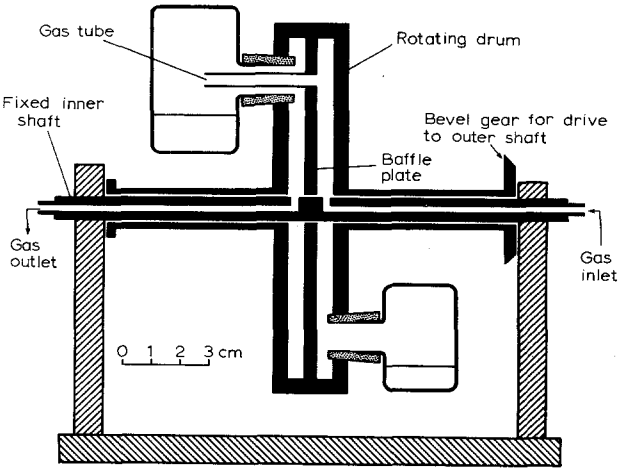

Fig. 3. Diagram showing alternative construction of rotator.

Figure 3 also shows an alternative arrangement for the drive to the outer shaft and for the ducts in the inner shaft. Either arrangement of drive and ducts, as shown in figures 2 and 3, can be combined with either type of baffle plate.

Comparison of rotator and roller systems. The table shows the results obtained in an experiment to compare the rotator system with a roller system employing sealed bottles. All 8 bottles were of the type and size (capacity 30 ml) shown in figure 2 and each contained 5 ml of rat serum. 4 of the bottles were incubated on a rotator as in figure 2 with a continuous supply of 20% O_2/5% CO_2/75% N_2. The other 4 bottles were gassed initially with the same gas mixture and then stoppered and incubated on motor-driven horizontal rollers. 2 of the bottles in each group contained rat embryos (5 embryos per bottle) explanted at the early somite stage (10.5 days gestation) by methods described previously[12].

After 24 h incubation, all the embryos had developed normally to early limb-bud stages with a good heartbeat and blood circulation. The total protein content of the 5 embryos (with their membranes) in each bottle increased from about 0.5 mg initially to about 2.5 mg at the end of the 24-h culture period. There was no significant difference between the embryos grown in the rotator bottles and those in the roller bottles, but differences were found in the pO_2, pCO_2, pH and osmolarity of the culture serum.

pO_2. Without embryos, the serum in the roller bottles showed a higher pO_2 than in the rotator (as would be expected, because the roller bottles were gassed and stoppered at room temperature, before incubation). But with embryos present, pO_2 in the roller bottles fell to well below that of the rotator bottles.

pCO_2 and pH. Without embryos, pCO_2 was lower, and pH correspondingly higher, in the serum in the roller bottles than in the rotator, presumably as a result of different equilibria established between dissolved CO_2, carbonates and bicarbonates. With embryos present, pCO_2 more than doubled in the roller bottles but remained constant in the rotator bottles; in both systems there was a fall in pH, but the fall was much greater, to about 6.95, in the roller bottles.

Osmolarity. The osmolarity of the serum in the rotator bottles was slightly lower (about 295) than in the roller bottles (about 300), probably because the gas flow to the rotator was humidified by bubbling through distilled water, resulting in a small net transfer of water vapour to the serum. The effect is probably insignificant for tissue growth, but could be avoided by humidifying the gas with a salt solution instead of distilled water.

Discussion. In the experiment described, tissues (i.e. somite-stage rat embryos) were chosen which grow equally well in both rotator and roller systems and could therefore be regarded as providing comparable levels of O_2 consumption and CO_2 production in each system. The results show that in the rotator system, pO_2 and pCO_2 in the culture medium remain constant, while in the sealed roller bottles, pO_2 and pCO_2 may undergo large changes. Furthermore, the fall in pH in the rotator bottles is much less than in roller bottles. For cell, tissue, organ and embryo cultures where pO_2, pCO_2 and pH levels are critical, and where it is necessary to define these levels closely, the rotator system with a continuous gas flow has clear advantages.

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2 J. Paul, Cell and Tissue Culture, 5th ed. Livingstone Ltd, Edinburgh and London 1975.
3 G.M. Hodges, in: Organ Culture in Biomedical Research. Ed. M. Balls and M. Monnikendam. Cambridge University Press, 1976.
4 C.R. Austin, ed. The Mammalian Fetus in vitro. Chapman and Hall, London 1973.
5 D.A.T. New, P.T. Coppola and S. Terry, J. Reprod. Fert. *35*, 135 (1973).
6 D.M. Kochhar, Teratology *11*, 273 (1975).
7 N.D. Agnish and D.M. Kochhar, J. Embryol. exp. Morph. *36*, 623 (1976).
8 E.M. Deuchar, J. Embryol. exp. Morph. *35*, 345 (1976).
9 D.A.T. New, P.T. Coppola and D.L. Cockroft, J. Embryol. exp. Morph. *36*, 133 (1976).
10 M.K. Sanyal, 1978.
11 S.K.L. Buckley, C.E. Steele and D.A.T. New, Devl Biol. *65*, 396 (1978).
12 D.A.T. New, in: Methods in Mammalian Embryology. Ed. J.C. Daniel. W.H. Freeman & Co. 1971.

PRO EXPERIMENTIS

Immunological tests for LH and FSH in urine

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Summary. The addition of 100 g/l NaCl and 200 mg/l nitrofurantoin to 24-h urine samples destined for determination of LH and FSH with haemagglutination tests, proved to be an adequate preservative in comparison to chilling.

Immunological methods for detecting FSH and LH are relatively new. Haemagglutination inhibition (HAI) tests[1-3] are easy to perform, using erythrocytes sensitized with FSH or human chorionic gonadotrophin (HCG), respectively. Such erythrocytes may be agglutinated by an antiserum against FSH or HCG. Agglutination may be prevented by free FSH or HCG and, in the latter case, also by LH, which cross-reacts with the antiserum against HCG. Since HCG is found only in the urine of pregnant women and in some rare diseases (e.g. chorionepithelioma), the latter test may serve to determine LH in all other cases.

The HAI tests are designed to analyse urine, which, if the test cannot be carried out immediately after urination, should be kept in cold storage, or preferably frozen. Urine should not be kept in cold storage longer than 24 h. In view of the practical drawbacks of this method of preserving urine, we have looked for an alternative method, i.e. the addition of suitable preservatives. Substances with an aro-