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Amphibian Organ Culture

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1. *Introduction*

Organ cultures, in which cells and tissues retain their three-dimensional relationships in vitro, have been widely used to study the control mechanisms of cell differentiation, cell proliferation and cell function in a variety of tissues from many species. However, although organ cultures have been useful in studies on *morphogenesis* in avian and mammalian embryonic and foetal tissues, the method has been less successful in tackling the problem of *morphostasis* – the maintenance of normal differentiation and function in the adult. This is because, although fragments of some adult mammalian organs, such as lung, prostate gland and skin, survive well in organ culture, fragments of adult mammalian liver, kidney, pancreas and spleen do not. Conversely, fragments of adult amphibian organs survive well in long-term organ culture. This means that adult amphibian organ cultures are of great potential importance in seeking solutions to the problem of morphostasis and the related problem of *carcinogenesis*.

After discussing the scope of this review, we shall summarize past work on amphibian organ culture, the methods used, some possible reasons for the success of the organ culture technique with amphibian tissues, and future prospects for research.

2. *Definitions and exclusions*

The definitions given by FEDOROFF[1] in 'Proposed Usage of Animal Tissue Culture Terms' are used here. The term 'culture' is applicable only when cells, tissues or organs have been maintained or grown in vitro for more than 24 h, and 'organ culture' is defined as the maintenance or growth of tissues, organ primordia, or the whole or parts of an organ in vitro in such a way that may allow differentiation and preservation of the architecture and/or function, whereas in 'cell culture' the cells are no longer organized into tissues.

We have included in this review studies where the culture medium provides nutrition for larval (tadpole) and adult tissues and organs in vitro. Experiments on prehatching tissues are excluded, since amphibian eggs contain the factors necessary for development to the hatching stage independent of the external environment and each cell has an endogenous food supply. Methods used in culturing tissues of embryonic and early larval stages have been summarized by JACOBSON[2]. Cell cultures and explant cultures (in which an excised tissue or organ fragment is used to initiate a cell culture) are also excluded. Amphibian cell culture has recently been reviewed by FREED and MEZGER-FREED[3] and by RAFFERTY[4]. The recent paper by SOLURSH and REITER[5] is a good example of what we consider to be explant culture and not organ culture, as they looked only at cells emerging from *Xenopus* liver explants and not at cells within the fragments.

3. *Review of past work involving amphibian organ culture*

Previous research using amphibian organ culture is summarized in Table I. Many of the articles referred to concern the retention of normal structure during long periods in vitro – a subject to which we return in sections 4, 5 and 6. Other problems tackled will now be discussed.

1. *Regeneration*

One of the major features of Amphibia is their ability to regenerate lost organs and appendages. Organ culture studies have helped in identifying the factors controlling regeneration in vivo.

[1] S. FEDOROFF, Expl Cell Res. *46*, 642 (1967).
[2] A. G. JACOBSON, in *Methods in Developmental Biology* (Crowell, New York 1967).
[3] J. J. FREED and L. MEZGER-FREED, Meth. Cell Physiol. *4*, 19 (1970).
[4] K. A. RAFFERTY, in *Physiology of the Amphibia* (Academic Press, New York in press), vol. 2.
[5] M. SOLURSH and R. S. REITER, Z. Zellforsch. *128*, 457 (1972).

Table I. Amphibian organ culture: species and organs used, and problems studied

Organ or tissue	Problem	Species[a]	Authors (date)
1. Skin	Effects of thyroxine and prolactin on skin	*D. viridescens*	GRANT and COOPER (1965)[6]
	Thyroxine, cell division and skin gland development	*X. l. laevis*	MCGARRY and VANABLE (1969)[7]
	Collagenase and hyaluronidase production	*R. catesbeiana*	EISEN and GROSS (1965)[8]
	Dermis and epidermis in wound healing	*R. pipiens*	BERLINER (1969)[9]
	Mitotic activity	*X. l. laevis*	SIMNETT and BALLS (1969)[10]
2. Anuran tadpole tail	Regeneration of tail tips	*H. a. savignji*	STEFANELLI et al. (1959)[11]
		X. l. laevis	HAUSER and LEHMANN (1962)[12]
	Hormone induced tail regression	*R. catesbeiana*	LINDSAY et al. (1967)[13]
		R. pipiens	FLICKINGER (1963)[14]; LINDSAY et al. (1967)[13] DERBY (1968)[15]; GONA (1969)[16]; FRY (1972)[17]
		R. temporaria	TATA (1966)[18]
		X. l. laevis	SCHAFFER (1963)[19]; WEBER (1963)[20]; EECKHOUT (1966)[21]; HICKEY (1971)[22]; ROBINSON (1972)[23]
3. Urodele limb regenerates	Formation of blastema	*T. cristatus*	LECAMP (1948)[24]
		D. viridescens	BROMLEY and ANGUS (1971)[25]
	Collagenase production by blastema	*D. viridescens*	GRILLO et al. (1968)[26]
	Differentiation of blastema	*D. viridescens*	FIMIAN (1959)[27]
		A. maculatum	STOCUM (1968)[28]
		D. viridescens	JOHNSON-MULLER and BALLS (1970)[29]
4. Lens	Ionic levels	*B. b. bufo*	DUNCAN (1969)[30]
	Control of mitosis	*R. catesbeiana*	ROTHSTEIN (1968)[31]
		R. pipiens	GIERTHY and ROTHSTEIN (1971)[32]
		R. clamitans	GIERTHY et al. (1968)[33]
		B. marinus	
	Lens regeneration	*D. viridescens*	STONE and GALLAGHER (1958)[34]; EISENBERG-ZALIK and SCOTT (1969)[35]
		D. pyrrhogaster	EGUCHI (1967)[36]
		X. l. laevis	CAMPBELL and JONES (1968)[37]
5. Liver	Thyroxine and urea cycle enzyme activities	*R. catesbeiana*	BENNETT et al. (1969)[38]; COHEN (1970)[39]
	Vitellogenin synthesis	*X. l. laevis*	WALLACE and JARED (1969)[40]
	Glycogen accumulation	*D. picta*	BEAUMONT (1956)[41]
	Mitotic activity	*A. means*	MONNICKENDAM and BALLS (1972)[42]
		T. c. carnifex	MONNICKENDAM et al. (1970)[43]
		X. l. laevis	SIMNETT and BALLS (1969)[40]; BALLS et al. (1969)[44]
	Maintenance	*S. mexicanum*	FOOTE and FOOTE (1965)[45]
		P. waltii	BALLS et al. (1969)[44]
		R. pipiens	

Table I continued p. 3

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[7] M. P. MCGARRY qnd J. W. VANABLE, Devl Biol. *20*, 291 (1969).
[8] A. Z. EISEN and J. GROSS, Devl Biol. *12*, 408 (1965).
[9] J. BERLINER, Devl Biol. *20*, 544 (1969).
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[11] A. STEFANELLI, G. THERMES and R. MASIDDA, Rc. Semin. Fac. Sci. Univ. Cagliari *20*, 137 (1950).
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[18] J. R. TATA, Devl Biol. *13*, 77 (1966).
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[20] R. WEBER, in *Lysosomes* (CIBA Foundation Symposium; Churchill, London 1963).
[21] Y. EECKHOUT, Revue Quest. scient. *137*, 377 (1966).
[22] E. D. HICKEY, Wilhelm Roux' Arch. Entw Mech. Org. *166*, 303 (1971).
[23] H. ROBINSON, J. exp. Zool. *180*, 127 (1972).
[24] M. LECAMP, C. r. Acad. Sci. Paris *226*, 695 (1948).
[25] S. C. BROMLEY and D. J. ANGUS, Devl Biol. *26*, 652 (1971).
[26] H. C. GRILLO, C. M. LAPIERE, M. H. DRESDEN and J. GROSS, Devl Biol. *17*, 571 (1968).
[27] W. J. FIMIAN, J. exp. Zool. *140*, 125 (1959).
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[30] G. DUNCAN, Expl Eye Res. *8*, 315 (1969).
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[36] G. EGUCHI, Embryologia *9*, 246 (1967).
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[39] P. P. COHEN, Science *168*, 533 (1970).
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[41] A. BEAUMONT, C. r. Acad. Sci. Paris *243*, 676 (1956).
[42] M. A. MONNICKENDAM and M. BALLS, J. Cell Sci. *11*, in press (1972).
[43] M. A. MONNICKENDAM, J. L. MILLAR and M. BALLS, J. Morph. *243*, 676 (1970).

Table I continued from p. 2

Organ or tissue	Problem	Species[a]	Authors (Date)
6. Pancreas	Maintenance	*S. mexicanum*	FOOTE and FOOTE (1965)[45]
	Mitotic activity	*A. means*	MONNICKENDAM and BALLS (1972)[42]
		T. c. carnifex	MONNICKENDAM et al. (1970)[43]
		X. l. laevis	BALLS et al. (1969)[44]
7. Intestine	Mitotic activity	*T. c. carnifex*	MONNICKENDAM et al. (1970)[43]
	Maintenance	*X. l. laevis*	BALLS et al. (1969)[44]
8. Heart	Effect of pressure on beating	*R. pipiens*	LANDAU and MARSLAND (1952)[46]
	Beating and histology	*S. mexicanum*	FOOTE and FOOTE (1965)[45]
	Beating and ultrastructure	*T. torosa*	MILLHOUSE et al. (1971)[47]
	Effect of temperature on beating	7 Leptodactylid species	STEPHENSON (1968)[48]
9. Lung	Mitotic activity	*A. means*	MONNICKENDAM and BALLS (1972)[42]
		X. l. laevis	SIMNETT and BALLS (1969)[10]
	Maintenance	*P. waltlii; R. pipiens*	BALLS et al. (1969)[44]
10. Spleen	Mitotic activity	*A. means*	MONNICKENDAM and BALLS (1972)[42]
		X. l. laevis	SIMNETT and BALLS (1969)[10]; BALLS et al. (1969)[44]
	Maintenance	*S. mexicanum*	FOOTE and FOOTE (1965)[45]
		P. waltlii; T. cristatus; R. pipiens	BALLS et al. (1969)[44]
	Immune response	*X. l. laevis*	AUERBACH and RUBEN (1970)[49]
11. Kidney	Mitotic activity	*A. means*	MONNICKENDAM and BALLS (1972)[42]
		X. l. laevis	CHOPRA and SIMNETT (1969)[50]; SIMNETT and BALLS (1969)[10]
	Maintenance	*S. mexicanum*	FOOTE and FOOTE (1965)[45]
		P. waltlii	BALLS et al. (1969)[44]
	Induction of virus particles in tumour cells	*R. pipiens*	BREIDENBACH et al. (1971)[51]
12. Gonads	Maintenance	*P. waltlii*	FOOTE and FOOTE (1962)[52]
		S. mexicanum	FOOTE and FOOTE (1965)[45]
		T. cristatus	FOOTE and FOOTE (1957)[53]
		R. clamitans	FOOTE and FOOTE (1957)[54]
		R. pipiens	BALLS et a l. (1969)[44]
		X. l. laevis	SIMNETT and BALLS (1969)[10] BALLS et al. (1969)[44]
	Effects of hormones	*R. catesbeiana; X. l. laevis*	FOOTE and FOOTE (1959)[55]
		R. pipiens	BASU, NANDI and NANDI (1967)[56]
13. Miscellaneous	brain	*S. mexicanum*	FOOTE and FOOTE (1965)[45]
	adenohypophysis	*D. viridescens*	LIVERSAGE and LIIVAMAGI (1971)[57]

[a] Full names of species are given when first mentioned in the text or in the Appendix.

a) Tadpole tail regeneration. HAUSER and LEHMANN[12] found that *Xenopus laevis laevis* tail tips could be kept in good condition for at least a month in vitro. Fifteen days after amputation, 7 mm tail tips had developed 1 mm regenerates, whereas donor tadpoles had 4–5 mm regenerates at the site of amputation. Epidermis regenerated well but with irregular proliferations and vesicles at the amputation site; notochord and neural tube differentiated normally, but were considerably reduced in size; new blood capillaries developed and contained blood cells, but no differentiating myoblasts were seen in the isolated regenerates. HAUSER[58] later showed that a factor from the mid-brain stimulated regeneration.

b) Urodele limb regeneration. STOCUM[28] cultured forelimb regenerates of larval *Ambystoma maculatum* with and without stump tissues. He found that

[44] M. BALLS, J. D. SIMNETT and E. ARTHUR, in *Biology of Amphibian Tumors* (Springer Verlag, New York 1969).

[45] F. M. FOOTE and C. L. FOOTE, Trans. Ill. State Acad. Sci. *58*, 164 (1965).

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[52] C. L. FOOTE and F. M. FOOTE, J. Embryol. exp. Morph. *10*, 465 (1962).

[53] C. L. FOOTE and F. M. FOOTE, Anat. Rec. *127*, 415 (1957).

[54] C. L. FOOTE and F. M. FOOTE, Trans. Ill. State Acad. Sci. *50*, 243 (1957).

[55] C. L. FOOTE and F. M. FOOTE, Archs. Anat. microsc. Morph. exp. *48*, 71 (1959).

[56] S. L. BASU, J. NANDI and S. NANDI, J. exp. Zool. *162*, 245 (1967).

[57] R. A. LIVERSAGE and L. LIIVAMAGI, J. Embryol. exp. Morph. *26*, 443 (1971).

histologically undifferentiated cone stage blastemas could differentiate precartilage and striated muscle in vitro, but mature muscle was formed more frequently in cultures of later stage regenerates which contained cartilage at the time of explantation. Skeletal differentiation did not proceed beyond the precartilage stage in vitro, and inclusion of the stump tissues in explants did not enhance in vitro differentiation and may have been detrimental to the differentiation of the blastema. Cone, paddle and digit stages of forelimb regenerates of adult *Diemictylus viridescens* have also been cultured[29] and cone stages developed precartilage condensations in vitro. Cartilage in digit stages was well-maintained in vitro and thyroxine improved these cultures. However, when thyroxine was added to paddle stage regenerates, they tended to revert to a blastema-like state, containing undifferentiated cells. BROMLEY and ANGUS[25] studied regeneration in vitro in freshly amputated *D. viridescens* limbs, and found no obvious differences from regeneration in vitro. The wound was rapidly closed by migrating epidermis, which then thickened to form an epidermal cap. Muscle layers began to break down to block-like fragments with nuclei resembling those of blastema cells.

c) Lens regeneration. In larval and adult urodeles, complete removal of the lens is followed by the formation of a new lens by a cell population which is derived from the dorsal part of the iris epithelium. This is known as Wolffian lens regeneration, and is one of the few cases known where cells can lose their original tissue specificity and acquire another tissue specificity. Organ culture studies of isolated iris and of lens regenerates have been used to determine the role of the eye, and in particular the neural retina, in controlling lens regeneration. STONE and GALLAGHER[34] cultured iris membranes from eyes of normal adult *D. viridescens* and found no indications of lens regeneration during 28 days in vitro. When cultured irises were transferred to freshly lentectomized eyes, some were transformed into lenses. EISENBERG-ZALIK and SCOTT[35] cultured regenerating lenses from adults of the same species at various stages after lentectomy, to find out the extent to which differentiation could be maintained in vitro. Their conclusion was that lens regenerates in vitro were not capable of sustained development, but could only continue those processes initiated in vivo. Presumably, in both these experiments, various factors, absent from the culture medium, were required. EGUCHI[36] cultured irido-corneal complexes of *Diemictylus pyrrhogaster* (before and after lentectomy) to study the role of the retina in lens regeneration. Retinal explants had no effect on cultures of early stage regenerates. When later stage regenerates were cultured, fibre production continued, but no lens epithelium was formed. However, when retina from normal or lentectomized eyes was present, normal lens formation occurred in vitro. Thus, retinal explants were able to provide at least some of the factors missing from the EISENBERG-ZALIK culture system. CAMPBELL[59] found that when *X. l. laevis* tadpoles were lentectomized, they could regenerate new lenses from the anterior corneal epithelium, the iris or the neural retina. Cultured tadpole corneas[37], with or without pericorneal ectoderm, produced well-defined condensations of elongated cells after 24 h in vitro. During 7 days in culture, the condensate was transformed into a lentoid, and the remaining cells migrated to form a hemispherical dome of cells, the condensate usually forming part of the wall, enclosing a fluid-filled space. Lens antigens were found only in the condensate, which developed into a histologically normal lens.

2. Control of cell division

In the adult amphibian lens in vivo only the lens epithelial cells of the germinative zone normally divide, and these give rise to the lens fibres. In adult *Rana catesbeiana* kept at 24 °C the cell cycle time was found to be 83 days[60], with an S period of 100 h and a G2 period of 11 h. The time for mitosis was not determined, but over 90% of the total time was a prolonged G1 period. When lenses were cultured at 24 °C, many of the non-germinative epithelial cells began to proliferate and passed through DNA synthesis and mitosis in synchrony. The non-germinative cells began to incorporate ^{3}H-thymidine into DNA at 48 h after explantation, and mitosis was first observed at 72 h[61]. After prolonged culture, there was a second peak in DNA synthesis followed by a second wave of mitosis, and the cell cycle time, estimated from the period elapsing between two successive bursts of DNA synthesis involving the same cells, was 177 to 190 h[60]. This induced cell synchrony has been used by ROTHSTEIN[61] and his associates to investigate biochemical events leading to division in the lens epithelium. The results obtained from studies on the effects of inhibitors of DNA, RNA and protein synthesis and mitosis were the same as in experiments using injured lenses in vivo.

VANABLE et al.[7] looked at the development of subepidermal skin glands of *X. l. laevis*, which first appear at metamorphosis. They found that thyroxine increased mitotic activity and stimulated the development of skin glands in tadpole skin in vitro. Fluoridine-5'-deoxyribose, which inhibits mitosis, also inhibited skin gland formation, and their results suggested that skin glands develop by the division of single precursor cells.

[58] R. HAUSER, Wilhelm Roux' Archiv Entw Mech Org. *156*, 404 (1965).
[59] J. C. CAMPBELL, Anat. Rec. *145*, 214 (1963).
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SIMNETT and BALLS[10] looked at the mitotic activity of tissues from immature *X. l. laevis* in vitro and found that mitotic activity in liver, kidney, lung epithelium and ovarian follicle cells was much higher than in vivo; in epidermis and spleen it was much lower, and in testis there was no apparent change in the number of meiotic cells. MONNICKENDAM, MILLAR and BALLS[43] found that the proliferative activity of cultured liver and pancreas from adult *Triturus cristatus carnifex* increased, whilst that of intestinal epithelium fell dramatically. MONNICKENDAM and BALLS[42], using adult *Amphiuma means*, found large increases in the proliferative activity of pancreas and kidney in vitro, but no changes in the mitotic activity of liver, spleen and lung.

3. *Metamorphosis*

Most of the work discussed here has used in vitro systems to see whether thyroxine and other compounds which induce metamorphosis when injected into animals or added to tank water, also have a direct effect on individual organs or organ fragments. Isolated anuran tadpole tails begin to shorten when thyroxine is added to the medium, in the same way as tail resorption occurs in natural or thyroxine-induced metamorphosis. LINDSAY et al.[13] compared induced shortening of *R. catesbeiana* and *Rana pipiens* tail tips under various conditions and, using thyroxine analogues, found that the selectivity of response observed in vivo was maintained in vitro. FRY[17] looked at the effects of temperature on thyroxine-induced shortening of *R. pipiens* tail tips and found that the rate of shortening was temperature dependent. At lower temperatures (5–10 °C), the rate of hormone uptake from the medium was reduced, and processes subsequent to hormone entry were also affected. DERBY[15, 62, 63] used tail fin discs, devoid of muscle and cut from *R. pipiens* tails, for a quantitative assay of response to thyroxine. There was a quantitative relationship between hormone concentration and the rate of shrinkage of tissue from premetamorphic animals; control discs from metamorphosing animals shrank spontaneously, but the rate of shrinkage was increased when thyroxine was added. Tadpole pituitary glands implanted into tail discs inhibited thyroxine-induced resorption, and also inhibited the spontaneous resorption of discs from older animals. Pituitary glands from different stages differed in their capacity to inhibit hormone-induced resorption; those from animals in early metamorphosis were most effective, those from climax animals were somewhat less effective, and froglet glands were least effective. Thyroxine did not induce shrinkage of discs injected with mammalian prolactin or growth hormone, but those injected with follicle stimulating hormone responded normally. Light and electron microscope studies by GONA[16] showed that the histological changes observed in vivo also occurred in thyroxine-treated tail discs in vitro. Changes in the activities of acid hydrolases and lactate dehydrogenases were the same in thyroxine-treated discs as those observed in vitro[64].

TATA[18] used *Rana temporaria* tail tips and found that during triiodothyronine-induced regression the activities of acid phosphatase, cathepsin and ribonuclease increased and there was increased synthesis of RNA and protein. Actinomycin D, puromycin and cycloheximide inhibited increases in RNA and protein synthesis and there was a good correlation between the inhibition of synthesis and inhibition of tail regression. Isolated tail tips from *X. l. laevis* shrank when thyroxine[20] or triiodothyronine[19] were added to the medium. The lag period between addition of hormone and tail regression was much shorter in tails from later stages than in tails from earlier stages. Thyroxine increased nitrogen loss from tails and the increase in cathepsin activity was far more marked in vitro than in vivo. Actinomycin D and puromycin inhibited thyroxine-induced tail regression[21]. HICKEY[22] found that thyroxine caused significant decreases in DNA and protein content of tails after 3 and 6 days, respectively. There were significant increases in the activities of cathepsin, DNAase and acid phosphatase in treated tails compared with control tails, and these increases occurred 2 days before detectable loss of protein. ROBINSON[23] looked at qualitative and quantitative changes in acid phosphatase during thyroxine-induced tail regression and found the same types of changes in activity, pH optimum, heat-sensitivity and relative amounts of two electrophoretic forms of the enzyme as occurred in spontaneous metamorphosis.

During metamorphosis, urea replaces ammonia as the main nitrogenous waste product, and there are large increases in the activities of urea cycle enzymes in the liver. The experiments described here were carried out to see whether similar increases could be induced directly by adding thyroxine to liver cultures. N-acetyl-L-glutamate-dependent carbamyl phosphate synthetase, which catalyses the production of carbamyl phosphate from ammonia and carbon dioxide, was studied[65] in liver cubes from *R. catesbeiana* tadpoles and from frogs. Thyroxine in the medium increased the amount of carbamyl synthetase activity in liver cubes from premetamorphic and metamorphosing tadpoles and from adult frogs, whereas cortisol, diiodotyrosine, L-thyronine, insulin, growth hormone, adrenalin, cyclic adenosine monophosphate, premetamorphic tadpole serum, serum from metamorphosing tadpoles and frog serum had no effect. The thyroxine-

[62] A. DERBY and W. ETKIN, J. exp. Zool. *169*, 1 (1968).
[63] A. DERBY, J. exp. Zool. *173*, 319 (1970).
[64] P. GREENFIELD and A. DERBY, J. exp. Zool. *179*, 129 (1972).
[65] G. E. SHAMBAUGH, J. B. BALINSKY and P. P. COHEN, J. biol. Chem. *244*, 5295 (1969).

induced increase in enzyme activity was largely due to the conversion of inactive, non-immunoprecipitable precursors to active, immunoprecipitable enzyme, and also to the increased synthesis of enzyme from amino acids. There was no lag period between the addition of thyroxine and increased levels of carbamyl synthetase activity in vitro, whereas in vivo there was a marked lag, and it has been suggested that a repressor system operates in vivo which is inoperable in vitro. Ornithine transcarbamylase catalyses the reaction between ornithine and carbamyl phosphate to form citrulline, and thyroxine added to *R. catesbeiana* liver organ cultures increased the activity of the enzyme during the first 48 h in vitro, after which it remained constant[38]. Triiodothyronine also stimulated enzyme activity. Thyroxine treatment also increased the rates of incorporation of ^{3}H-lysine and ^{3}H-valine into protein. The third enzyme which has been studied in organ culture is glutamate dehydrogenase[66], a mitochondrial enzyme which is not part of the urea cycle. However, it can be regarded as an accessory to the cycle since it fixes ammonia to form glutamate, and this ammonia group can be passed on into the urea cycle. A small increase in enzyme activity occurred in untreated liver from premetamorphic tadpoles during 48 h in vitro, and thyroxine, triiodothyronine and triiodothyropropionate stimulated the encrease in activity by increasing the de novo synthesis of immunoprecipitable enzyme. Cortisol, insulin, growth hormone, adrenalin, acetylcholine, and serum from premetamorphic and metamorphosing tadpoles and adult frogs had no effect. Actinomycin D and puromycin decreased the de novo synthesis of enzyme in vitro, but both inhibitors, with or without thyroxine, increased the total enzyme activity as they also inhibited the breakdown of enzyme. Further studies[67] have shown that liver cubes continued urea biosynthesis for at least 48 h in vitro and there was a correlation between the rate of urea synthesis and carbamyl phosphate synthetase levels. The biosynthesis of urea from carbon dioxide and ammonia requires the integrated function not only of the mitochondrial enzymes carbamyl phosphate synthetase and ornithine transcarbamylase, but also the extramitochondrial enzymes argininosuccinate synthetase, argininosuccinase and arginase, which have not yet been studied in vitro.

4. *Vitellogenin synthesis*

Adult *X. l. laevis* liver cultures have been used in a study of the serum lipid phosphoprotein, vitellogenin, which occurs in large quantities in oocytes. Vitellogenin was found after ovulation in the serum of mature female toads which had been induced to ovulate by the injection of human chorionic gonadotrophin (HCG). Vitellogenin was also found in the serum of males after injections of oestrogens. There was no accumulation of vitellogenin in the serum of HCG-treated females, but the rate of loss from the serum of males and ovariectomized females was very low[68]. Short-term (3 h) incubation of various tissues showed that only liver from HCG-treated females or oestrogen-treated males synthesized and released vitellogenin into the external medium. Organ cultures of liver from normal males released no vitellogenin into the medium during the 4-day culture period, liver from normal females released a little vitellogenin at first, whilst liver from HCG-treated females and oestrogen-treated males rapidly released vitellogenin into the medium throughout the culture period[40]. The significance of these observations is that they show that the yolk proteins are not being synthesized by the egg cytoplasm or by the follicle cells[69], but by the liver.

4. *Methods used in amphibian organ culture*

1. *Culture methods.* Organ culture methods have been discussed in detail by PAUL[70] and MERCHANT et al.[71]. Three main techniques have been used in amphibian organ culture (Table II): a) Cultures submerged in liquid medium. This is the simplest and most commonly used method, which is far more successful with amphibian tissues than with mammalian tissues. The cultures are often shaken in order to facilitate medium circulation and gaseous exchange. We maintain cultures in 10 ml medium per capped 25 ml conical flask, shaken in a linear, simple harmonic shaker at a frequency of about 2 cycles per second[42].

b) Culture at a gas-liquid interface involves using rafts of filter paper, lens paper or Millipore filter which float on the liquid medium, or rigid platforms of metal gauze to support the tissue. The latter method is less troublesome to use, but both methods suffer from the drawback that the 'gas-side' and 'liquid-side' of the cultured fragments differ, the former in direct contact with gaseous oxygen and with reduced access to medium, the latter having ready access to medium but less access to oxygen.

c) Cultures on a solid substrate, which may be complex medium, physiological saline, embryo extract and/or serum solidified with agar, collagen, gelatin or a

[66] J. B. BALINSKY, G. E. SHAMBAUGH and P. P. COHEN, J. biol. Chem. *245*, 128 (1970).
[67] G. E. SHAMBAUGH, S. H. KANG and P. P. COHEN, J. biol. Chem. *245*, 4028 (1970).
[68] R. A. WALLACE and D. W. JARED, Can. J. Biochem. *46*, 953 (1968).
[69] W. ANDREW, *Textbook of Comparative Histology* (Oxford University Press, New York 1969).
[70] J. PAUL, *Cell and Tissue Culture*, 4th edn. (Livingstone, Edinburgh 1970).
[71] D. J. MERCHANT, R. H. KAHN and W. H. MURPHY, *Handbook of Cell and Organ Culture* (Burgess, Minneapolis 1965).

Table II. Methods used in amphibian organ culture

Method	Conditions of culture: Temperature (°C)	Gas phase	Basic medium	Serum etc.	Other information	Maximum time in culture (days)	Species[a]		Tissue or organ	Reference
1. Cultures submerged in liquid medium	25 (approx.)	air	Leibovitz L-15	foetal calf serum beef embryo extract	pH 7.4; 263 mOs/kg; not shaken	25	Urodela	*A. maculatum* L	forelimb regeneration blastema	STOCUM (1968)[28]
	25	air	Leibovitz L-15	foetal calf serum	shaken	28		*A. means* A	kidney, liver, lung, pancreas, spleen	MONNICKENDAM and BALLS (1972)[42]
	37	95% O_2, 5% Co_2	Amphibian Tyrode solution		not shaken	3 or more		*D. viridescens* A	forelimb regeneration blastema	DRESDEN and GROSS (1970)[74]
	25	air	Leibovitz L-15	foetal calf serum	230, 260 or 290 mOs/kg; not shaken	30		*D. viridescens* A	forelimb regeneration blastema	JOHNSON-MULLER and BALLS (1970)[29]
	16	95% air, 5% CO_2	CMRL-1415 ATM	foetal calf serum insulin	pH 7.2; 230 mOs/kg; not shaken	27		*D. viridescens* A	adenohypophysis	LIVERSAGE and LIIVAMAGI (1971)[57]
	25	air	Leibovitz L-15	foetal calf serum	shaken	14		*T. c. carnifex* A	intestine, liver, pancreas	MONNICKENDAM et al. (1970)[43]
	19 (approx.)	air	salt solutions		pH 7.5; not shaken	1	Anura	*B. b. bufo* A	lens	DUNCAN (1969)[30]
	not given	air	199	rabbit serum	pH 7.1–7.2; not shaken 225–235 mOs/kg	3		*B. marinus* A	lens	GIERTHY et al. (1968)[33]
	not given	air	Holtfreter or Niu and Twitty solution	chick embryo extract	glucose; not shaken	2		*D. picta* T	liver	BEAUMONT (1956)[41]
	not given	air	Holtfreter or Ringer solution		not shaken	7		*H. a. savignji* T	tail tip	STEFANELLI et al. (1959)[11]
	18–22	air	Holtfreter solution		not shaken	10		*R. catesbeiana* T	tail	LINDSAY et al. (1967)[13]
	30	95% air, 5% CO_2	Wolf and Quimby		shaken	6		*R. catesbeiana* T	liver	BENNETT et al. (1969)[38]
	24	air	Wolf and Quimby		shaken	6		*R. catesbeiana* T	liver	SHAMBAUGH et al. (1969)[86]
	16, 24, 30	air	Wolf and Quimby 199	rabbit serum	not shaken	5		*R. catesbeina* A	lens	ROTHSTEIN et al. (1965)[77]
	not given	air	199	rabbit serum	pH 7.1–7.2; not shaken 225–235 mOs/kg	90		*R. catesbeiana* A	lens	ROTHSTEIN et al. (1970)[84]
	not given	air	199	rabbit serum	pH 7.1–7.2; not shaken 225-235 mOs/kg			*R. clamitans* A	lens	GIERTHY et al. (1968)[33]
	20	air	Niu and Twitty		pH 7.8; not shaken	13		*R. pipiens* T	tail	FLICKINGER (1963)[14]
	18–22	air	Niu and Twitty Holtfreter solution		not shaken	10		*R. pipiens* T	tail	LINDSAY et al. (1967)[13]
	20	air	Hanks solution		not shaken	20		*R. pipiens* T	tail fin discs	DERBY (1968)[15]
	5, 10, 20, 16	air	Niu and Twitty		not shaken	20		*R. pipiens* T	tail	FRY (1972)[17]
	26 (approx.)	95% O_2, 5% CO_2	199		pH 7.4; not shaken	15		*R. pipiens* A	testis	BASU et al. (1967)[86]

Table II continued p. 8

Table II continued from p. 7

Method	Conditions of culture: Temperature (°C)	Gas phase	Basic medium	Serum etc.	Other information	Maximum time in culture (days)	Species[a]	Tissue or organ	Reference
	4, 10, 24, 33	air	199	rabbit serum	225–235 mOs/kg; not shaken	6	*R. pipiens* A	lens	GIERTHY and ROTHENSTEIN (1971)[32]
	20	air	Gey solution	chick embryo extract calf serum	shaken at intervals	8	*R. temporaria* T	tail	TATA (1966)[18]
	18–20	air	Holtfreter solution		pH 7.2; not shaken	12	*X. l. laevis* T	tail	WEBER (1963)[20]
	18	air	Holtfreter solution		not shaken	44	*X. l. laevis* T	tail	HAUSER and LEHMANN (1962)[12]
	not given	95% O_2, 5% CO_2	Trowell solution	foetal calf serum	pH 7.5–7.6; roller tube	7	*X. l. laevis* T	head and trunk skin	VANABLE and MORTENSEN (1966)[63]
	25–26	air	Niu and Twitty		pH 7.8; not shaken	3	*X. l. laevis* T	trunk region	CHOPRA and SIMNETT (1969)[50]
	15–27	air	Steinberg solution		pH 7.7; not shaken	18	*X. l. laevis* T	tail tip	HICKEY (1971)[22]
	21	air	Hanks solution		not shaken	8	*X. l. laevis* T	tail	ROBINSON (1972)[23]
	not given	air	Steinberg solution		not shaken	6	*X. l. laevis* T	forelimb	MCGARRY and VANABLE (1969)[7]
	20	air	Leibovitz L-15	*R. catesbeiana* serum	shaken	4	*X. l. laevis* A	liver	WALLACE and JARED (1969)[40]
	25	air	Leibovitz L-15	foetal calf serum	not shaken	30	*X. l. laevis* A	spleen	AUERBACH and RUBEN (1970)[49]
2. Cultures at a gas-liquid interface	18, 26	air		horse serum; chick embryo extract	raft of rayon acetate cloth	28	Urodela *D. viridescens* A	iris	STONE and GALLAGHER (1958)[34]
a) Floating rafts	24–25, 37	95% O_2, 5% CO_2	Amphibian Tyrope solution	human serum	raft of filter paper	5	Anura *R. catesbeiana* T	back and tail fin skin	GROSS and BRUSCHI (1971)[62]
	22–24	95% air, 5% CO_2	Steinberg solution	foetal calf serum	raft of Millipore filter	10	*R. pipiens* T	tail skin	BERLINER (1969)[9]
	26	air	K-free Tyrode soln Parker medium	calf serum chick embryo extract	raft of siliconized cellulose acetate	21	*X. l. laevis* T	tail	SCHAFFER (1963)[19]
b) Rigid platforms or grids	25	air	Leibovitz L-15	calf serum; foetal calf serum	raft of lens paper supported by square	7	Urodela *P. waltlii* A	kidney, liver, lung, spleen	BALLS et al. (1969)[44]
					grid of expanded	18	*T. cristatus* A	liver, spleen	
					titanum mesh	18	Anura *R. pipiens* A	liver, lung, ovary, spleen, testis	
	18, 25					18	*X. l. laevis* A	intestine, kidney, liver, lung, ovary, oviduct, pancreas, spleen, testis	
3. Cultures on a solid substrate	22	air	199	horse serum chick embryo extract	agar substrate	22	Urodela *D. pyrrhogaster* A	iris, regenerating lens, cornea, retina	EGUCHI (1967)[36]
	not given	air		chick embryo extract	chick plasma substrate	14	*D. viridescens* eft A	skin	GRANT and COOPER (1965)[6]

Table II continued p. 9

Table II continued from p. 8

Method	Conditions of culture: Temperature (°C)	Gas phase	Basic medium	Serum etc.	Other information	Maximum time in culture (days)	Species [a]	Tissue or organ	Reference
	27, 31, 37	90% O_2, 10% CO_2	Tyrode solution		pH 7.6 collagen substrate	4	*D. viridescens* A	forelimb regeneration blastema	GRILLO et al. (1968)[26]
	not given	air	salt solution	chick embryo extract	pH 7.4; plasma substrate	70	*D. viridescens* A	regeneration blastema	FIMIAN (1959)[27]
	25	95% air, 5% CO_2	199	foetal calf serum	agar substrate pH 7.0–7.4	15	*D. viridescens* A	iris, lens regenerate	EISENBERG-ZALIK and SCOTT (1969)[35]
	18–20	95% air, 5% CO_2	Holtfreter or Tyrode solution	chick embryo extract	agar substrate 214 mOs/kg; pH 7.2	10	*D. viridescens* A	regenerating forelimb	BROMLEY and ANGUS (1971)[25]
	not given	not given	Tyrode solution	chick embryo extract	agar substrate	57 L 21 A	*P. waltlii* L, A	trunk region of larva; gonads, mesonephros and associated ducts of metamorphosing larva; adult testis	FOOTE and FOOTE (1962)[52]
	21–24	air	Tyrode solution	chick embryo extract	agar substrate	21	*S. mexicanum* L	brain, heart, liver, mesonephros, ovary, pancreas, spleen, testis	FOOTE and FOOTE (1965)[45]
	25	95% air, 5% CO_2	Eagle medium	foetal calf serum chick embryo extract	chick plasma substrate	180	*T. torosa* A	heart	MILLHOUSE et al. (1971)[47]
	21	air		chick embryo extract blastema extract	plasma substrate	10 or more	*T. cristatus* A	forelimb, regeneration blastema	LECAMP (1948)[24]
	not given	not given	not given	not given	rayon strips on plasma substrate	42	*T. cristatus* A	ovary, testis	FOOTE and FOOTE (1957)[53]
	4–41	air	199	chick embryo extract	pH 7.4; cockerel plasma substrate	12	Anura 7 Leptodactylid species	heart	STEPHENSON (1968)[48]
	27, 37	95% O_2, 5% CO_2	Tyrode solution		pH 7.2–8.2; collagen substrate		*R. catesbeiana* T	gill, gonad, gut, heart, notochord, tail, skin, kidney, liver, muscle, pancreas	EISEN and GROSS (1965)[8]
	not given	air		chick embryo extract	rayon acetate fabric; cock plasma substrate	28	*R. catesbeiana* T	ovary, testis	FOOTE and FOOTE (1959)[55]
	24–26	air		chick embryo extract *Rana* tissue extract	pH 7.2–7.4; agar substrate	29	R. *clamitans* T	ovary, testis	FOOTE and FOOTE (1957)[54]
	9–12	air	Holtfreter solution	chick embryo extract	pH 7.2–7.4; gelatin and chick plasma substrate	several days	*R. pipiens* T	heart	LANDAU and MARSLAND (1952)[46]
	7.5, 11.5, 15.5	air	Leibovitz L-15		agar substrate	98	*R. pipiens* A	renal adenocarcinoma	BREIDENBACH et al. (1971)[51]
	not given	air		chick embryo extract	rayon acetate fabric cock plasma substrate	28	*X. l. laevis* T	kidney, ovary, testis	FOOTE and FOOTE (1959)[55]
	not given	air	199		fowl plasma substrate	7	*X. l. laevis* T	cornea, pericorneal ectoderm	CAMPBELL and JONES (1968)[37]

[a] Full Latin names of species are given when first mentioned in the text or in the Appendix; A = adult; T = tadpole; L = larva.

plasma clot. This method suffers from the same disadvantage as the raft or platform method, and, in addition, the solid substrate reduces diffusion, so metabolites are less readily available to organ fragments. There is also the problem that medium will be exhausted and waste products will accumulate in the immediate vicinity of fragments. The collagen gel assay for collagenase[8] depends on the limited rate of diffusion of activity through the gel.

Amphibian material clearly does not normally require the use of complex Trowell culture chambers or expensive disposable dishes and grids.

2. *Basic media.* The basic media used fall into two main categories: a) physiological salt solutions, such as Ringer, Holtfreter, Niu and Twitty, Steinberg, Hanks, Tyrode or Gey (used mainly for isolated tail tips). In some experiments metabolites are added in the form of plasma, serum or embryo extract.

b) complex media, such as medium 199, Eagle's medium, medium CMRL-1415, Parker's medium, Trowell's medium, Wolf and Quimby's amphibian culture medium or Leibovitz L-15 medium.

Details of these solutions and media may be found in the papers referred to in Table II, in JACOBSON's[2] review or in PAUL's[70] book. Most of the complex media are buffered by bicarbonate ions used with increased CO_2 levels. However, raising the CO_2 tension may be detrimental to amphibian tissues, and there are two ways of avoiding this problem. Firstly, media may be buffered with one of the zwitterion buffers[72], which have been found suitable for a number of mammalian cell lines[73] and organ cultures[74]. We have found[75] that cells of *X. l. laevis* lines EAX1 and EAX2[76] grow in media containing 70% MEM-Autopow or BME-Autopow (Flow Laboratories, Irvine, Scotland) buffered with 14 m*M* HEPES (N-2-hydroxyethyl-piperazine-N'-ethane-sulphonic acid, Calbiochem, London), 20% water, 10%foetal calf serum and antibiotics. Cell morphology and growth rates were similar to those in Leibovitz L-15 medium. Primary cell cultures from young adult *X. l. laevis* kidney, liver and muscle were also successfully established in these media. Media buffered with HEPES should be equally suitable for amphibian-organ cultures. The alternative is to use Leibovitz L-15 medium[77], which was recommended as the most suitable basic medium for amphibian cell culture in two recent reviews[3,4], and has been widely used in amphibian organ culture research (Table II). This medium is buffered with free-base amino-acids, and is incubated in free gaseous exchange with the atmosphere. An obvious disadvantage of Leibovitz L-15 medium is in experiments where labelled amino-acids are added. The medium suggested for amphibian cell culture by WOLF and QUIMBY[78] seems unnecessary, since there is no evidence that amphibian cells or tissues normally require media fundamentally different from those used with avian or mammalian material.

3. *Serum, embryo extracts, hormones and other additives.* WALLACE and JARED[40] used medium containing 10% serum from male *R. catesbeiana*, which formed a thin clot around the liver fragments and held them in place. Under these conditions, with labelled leucine and sodium dihydrogen phosphate in the medium, twice as much labelled protein was released into the medium as from fragments cultured in commercially-supplied foetal calf serum. Nevertheless, we do not recommend the use of amphibian serum on a large scale, since it is expensive, obtainable from each animal in only small amounts, and, in any case, we do not feel that the ever-decreasing world population of amphibians should be decimated for such a purpose. Production of serum from the larger species might be considered as part of the service provided by the Michigan Amphibian Facility.

A wide range of other sera has been used in cultures. In some cases, serum seems to be essential, in others it does not. GROSS and BRUSCHI[79] used 10% normal human serum for *R. catesbeiana* skin cultures, and found that tissue fragments disintegrated in the absence of serum into a suspension of living cells and epithelial sheets showing nearly complete mesenchymal dissolution. When serum was present, the cut edges of tissue explants healed by epithelial migration, and fragments remained as intact, completely epithelialized structures. The amount of hydroxyproline released from explants and its specific activity were greatly reduced in the presence of serum, which seemed preferentially to protect newly-synthesized collagen from degradation. NAGAI et al.[80] found that bovine serum greatly reduced *R. catesbeiana* skin collagenase production in liquid media, whereas gelatin or collagen had no effect on the time of appearance or the total amount of enzyme activity. BERLINER[9] observed that foetal calf serum was essential for the long-term survival of *R. pipiens* tadpole skin cultures. HICKEY[22], using isolated tail tips of *X. l. laevis*, found that diluted medium 199, with or without 10% horse serum, and glucose solutions did not prevent the initial decrease in nitrogen content during wound healing, nor was survival improved; these media increased the frequency of bacterial and fungal contaimination.

ROTHSTEIN et al.[81] found that DNA synthesis and mitosis of *R. catesbeiana* lens epithelium cells did not

[72] N. E. GOOD, G. D. WINGET, W. WINTER, T. N. CONNOLLY, S. IZAWA and R. M. M. SINGH, Biochemistry *5*, 467 (1966).
[73] J. D. WILLIAMSON and P. COX, J. gen. Virol. *2*, 309 (1968).
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[77] A. LEIBOVITZ, Am. J. Hyg. *78*, 173 (1963).
[78] K. WOLF and M. C. QUIMBY, Science *144*, 1578 (1964).
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[80] Y. NAGAI, C. M. LAPIERE and J. GROSS, Biochemistry *5*, 3213 (1966).
[81] H. ROTHSTEIN, A. WEINSIEDER and N. FREEMAN, Experientia *26*, 1242 (1970).

occur in diluted 199 medium, but did occur in Wolf and Quimby's medium, which contains 10% foetal calf serum and 10% whole egg ultrafiltrate. ROTHSTEIN et al.[81] observed much more mitotic activity in *R. catesbeiana* lenses cultured in diluted medium 199 containing 20% rabbit serum than in serum-free medium. Lenses maintained without serum survived for up to 2 months, while with serum they survived for at least 3 months. GIERTHY and ROTHSTEIN[32], looking at the migration of *R. pipiens* lens epithelial cells in vitro, found that although migration occurred in the absence of serum, mitosis did not. CAMPBELL and JONES[37] used a 2% gelatin solution as supporting medium in place of fowl plasma for *X. l. laevis* tadpole cornea cultures, and observed a marked fall in the incidence of the dome phenomenon. When no supporting substrate was used, hollow vesicles with local thickenings in the wall were produced.

BEAUMONT[41] found that liver cultures of *D. picta* tadpoles could accumulate glycogen even if chick embryo extract was not present in what he called a synthetic (i.e. defined) medium, although it is not clear exactly what was present in the medium. Organ cultures of *A. means* kidneys tended to develop central necrosis after 14 days in vitro. The extent of this necrosis was dependent on the medium; less necrosis developed in media with higher L-15 and serum levels[82].

FOOTE and FOOTE have used several different media, with many variants, to culture gonads from several species at different stages. Their general conclusions were that growth and maintenance were better on media containing components from natural sources (e.g. amphibian tissue extracts, cock plasma, chick embryo extract) than on any of the defined media (e.g. medium 199, medium 1066, White's nutrient solutions). However, when hormones (e.g. chorionic gonadotrophin, oestradiol, testosterone) were added to the media, whether natural or defined, both germ-cells and somatic cells were far better preserved. Testosterone caused sex reversal in ovaries from *R. catesbeiana* tadpoles[55], but did not affect ovaries from *X. l. laevis* tadpoles. Oestradiol had no effect on testes from *X. l. laevis* tadpoles. BASU et al.[56], who cultured adult *R. pipiens* testes, found that testosterone, which inhibited spermatogenesis in vivo, had no such effect in vitro. Most combinations of protein hormones resulted in improved maintenance compared with hormone-free media.

In general, thyroxine and triiodothyronine had the same effect on tissues (e.g. skin gland formation, tail regression, liver enzyme activity) in vitro as in vivo, with the exception of *D. viridescens* skin, where GRANT and COOPER[6] found that thyroxine caused glandular atrophy and degeneration of the epidermis within a few days. After 2 weeks in vitro the epidermis had lost most of its structural organization. In vivo, thyroxine treatment at first caused an increase in glandular activity and epidermal moulting. After 2 weeks, when animals had migrated to land, there were very few glands in the epidermis, which was composed of a thin layer of cells covered with several layers of stratum corneum. They found that prolactin-treated skin cultures were well-maintained for several weeks in vitro. BROMLEY and ANGUS[25] found that somatotrophin (which probably contained prolactin) improved the maintenance of *D. viridescens* forelimb epithelium, which was thicker than in hormone-free medium. Cortisone had no effect on the maintenance of epithelium.

Antibiotics are normally necessary additions to amphibian culture media, because the small size of most amphibians, their moist skin and habitats make it difficult to obtain completely sterile cultures. As a routine precaution, we use 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml Fungizone (Squibb) in our media.

4. *Osmolality*. There is very little information available on the osmolalities of amphibian sera, though it has been reported that the osmolality of *A. maculatum* body fluid is 258 mosmol/kg[28], that of *A. means* serum 223 mosmol/kg[42], and that of *X. l. laevis* serum 245 mosmol/kg[83]. STOCUM[28] found that regenerating forelimb blastemas of *A. maculatum* could survive in hypotonic media, but differentiated further only in isotonic media. Organ cultures of *A. means* liver and spleen survived better in hypotonic and isotonic media than in hypertonic media[42].

5. *pH*. HICKEY[22] looked at the effects of varying the pH of Niu and Twitty solution on isolated *X. l. laevis* tadpole tails, and found that within the range pH 6.2–8.9 there was no significant effect on viability, maintenance of nitrogen content or regenerate length. Although most amphibian cell lines are similar to mammalian and avian lines in that they grow optimally at pH 6.8–7.2, some grow better at a higher pH[3]. This suggests that an investigation of the effects of varying the pH of amphibian organ culture media might be rewarding.

6. *Gas phase*. Most amphibian organ cultures have been incubated in free gaseous exchange with air. In a few cases, 95% air/5% CO_2 or 95% O_2/5% CO_2 have been used, in conjunction with bicarbonate buffer. In general, the oxygen requirements of amphibian tissues are lower than those of mammals, and the solubility of oxygen is greater at the lower incubation temperatures used for amphibian organ cultures. However, in a few cases, central necrosis, presumably due to inadequate oxygenation, has been observed, and it might be assumed that higher oxygen tensions would improve

[82] M. A. MONNICKENDAM, Ph. D. Thesis, University of East Anglia (1972).
[83] M. BALLS and R. S. WORLEY, Expl Cell Res., in press.

survival. SIMNETT and BALLS[10] observed central necrosis in immature adult *X. l. laevis* liver and kidney fragments, but cultures incubated in an oxygen atmosphere did not fare any better. SHAMBAUGH et al.[65] also observed necrosis in liver cubes from *R. catesbeiana* tadpoles after 6 days in vitro, and found that incubating in an atmosphere of oxygen or using smaller cubes had no effect on the viability of cultured tissues, but viability was reduced by inadequate shaking. BENNETT et al.[38] also observed necrotic cells in cultured *R. catesbeiana* tadpole liver fragments. The areas of necrotic cells were larger in thyroxine-treated fragments than in untreated fragments. Central necrosis was also found in cultures of adult *T. c. cristatus* liver[43] and *A. means* kidney[42], whilst *A. means* liver appeared normal after 35 days in vitro. Thus, some factor other than anoxia may be responsible for necrosis in amphibian organ cultures.

7. *Temperature.* Comparatively little work has been done on the effects of temperature on the various systems studied in organ culture. Obviously, the optimum temperature will vary between species, depending on their geographical distribution and other factors, and the optimum temperature may not be the same for each organ or the same as that for the intact organism.

GROSS and LAPIERE[84] found that *R. catesbeiana* tail fin cultures degraded four times as much collagen and produced twice as much dialysable hydroxyproline at 37 °C as at 27 °C. LINDSAY et al.[13] observed that variations in incubation temperature (18–22 °C) affected the maintenance of control tail tip length, the lag period before thyroxine-treated tails shortened, and the magnitude of response to thyroxine in *R. catesbeiana* and *R. pipiens* tail tips. HICKEY[22], using *X. l. laevis* tail tips, found that viability of control tail tips was unaffected by differences in temperature in the range 15 °C to 27 °C. FRY[17], working with *R. pipiens* tail tips at 5 °C to 26 °C, showed that the lower the temperature the less effective was thyroxine at inducing tail shortening. She also showed that both the rate of thyroxine uptake by the tails and some subsequent processes in the tail were retarded at lower temperatures. GRILLO et al.[26], looking at collagenase production by *D. viridescens* regenerating blastemas, found no differences in the amount of collagenase produced or the amount of collagen lysed at 27 °C and 37 °C.

STONE and GALLAGHER[34] found that *D. viridescens* lenses survived much better at 18 °C than at 26 °C. In cultured *R. catesbeiana* lenses[61], DNA synthesis in lens epithelium began on day 2 at 24 °C and 30 °C, but on day 5 at 16 °C. Mitosis occurred after 93 h at 24 °C and after 74 h at 30 °C[75]. The migration of *R. pipiens* lens epithelial cells to the anterior pole of the lens was also temperature-dependent[32]. No migration occurred at 4 °C, and the rate of migration increased with temperature in the range 10 °C to 33 °C.

SHAMBAUGH et al.[65], incubating *R. catesbeiana* tadpole liver cubes at 24 °C and 30 °C, found that at 30 °C there was a rapid decline in carbamyl phosphate synthetase compared with that in cubes incubated at 24 °C. However, the enzyme levels in cubes from tadpoles kept at 30 °C were twice those from tadpoles kept at 24 °C. BREIDENBACH et al.[51] cultured *R. pipiens* kidney slices for 14 weeks at 7.5 °C, 11.5 °C and 15.5 °C, but did not comment on any differences in maintenance at the different temperature. BALLS et al.[44] found that the mitotic incidences of spleen and pancreas fragments from adult *X. l. laevis* on culture day 7 were significantly higher at 25 °C than at 18 °C.

STEPHENSON[48], looking at the maintenance of heart beating in hearts from 7 frog species from a range of latitudes, has carried out a thorough study on the effect of varying the culture temperature. She found differences in the highest temperatures at which the different hearts survived – hearts from tropical species survived far better and far longer at higher temperatures (41 °C). All the hearts remained viable at 4 °C and although hearts from some tropical species stopped beating at this low temperature, they resumed beating when transferred to higher temperatures.

5. *The value of amphibian organ culture*

1. *Advantages of amphibians as donors of material for organ culture.* There are many factors which make amphibians particularly suitable for organ culture studies. First, there is a wide range of anuran and urodele species, and many are readily available and easy to maintain in the laboratory[85–87]. As a result of the increase in research using amphibians, an Amphibian Facility has been established at the University of Michigan[88] to develop defined strains of amphibian species, with the eventual aim of supplying other research departments. Secondly, amphibians have been pre-eminent in vertebrate experimental embryology, because eggs, embryos and larvae are easily obtained in vast numbers, are easy to operate on, able to recover from major surgical operations, and tolerant of a wide range of environmental conditions. As a result, a great deal is known about gametogenesis, embryogenesis, morphogenesis and metamorphosis in amphibians. Moreover, urodeles, in being able to regenerate whole appendages, are able to undertake large scale morphogenesis as adults.

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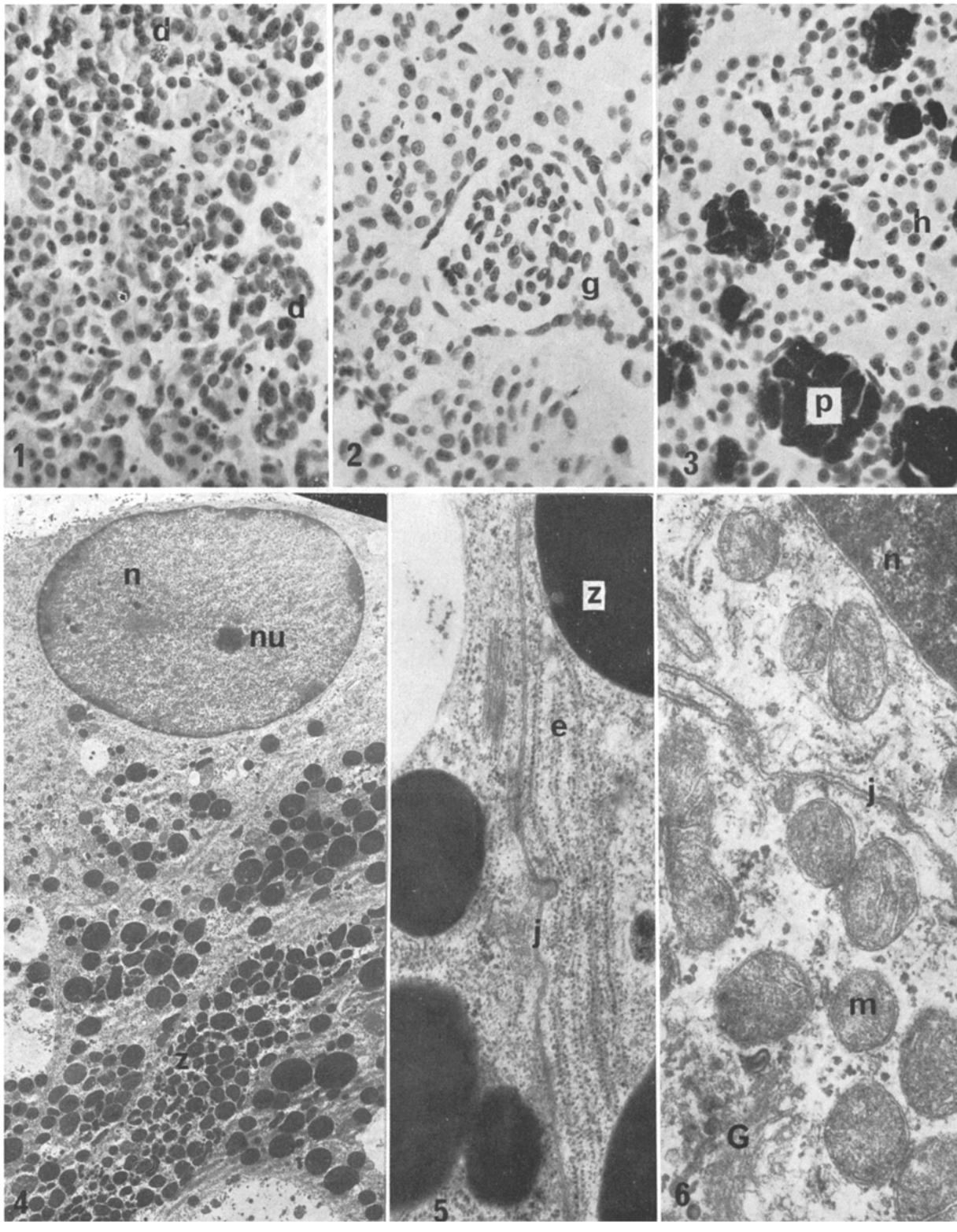


Fig. 1. *A. means* pancreas, culture day 21, showing acinar cells and mitotic figures (d). ×150.

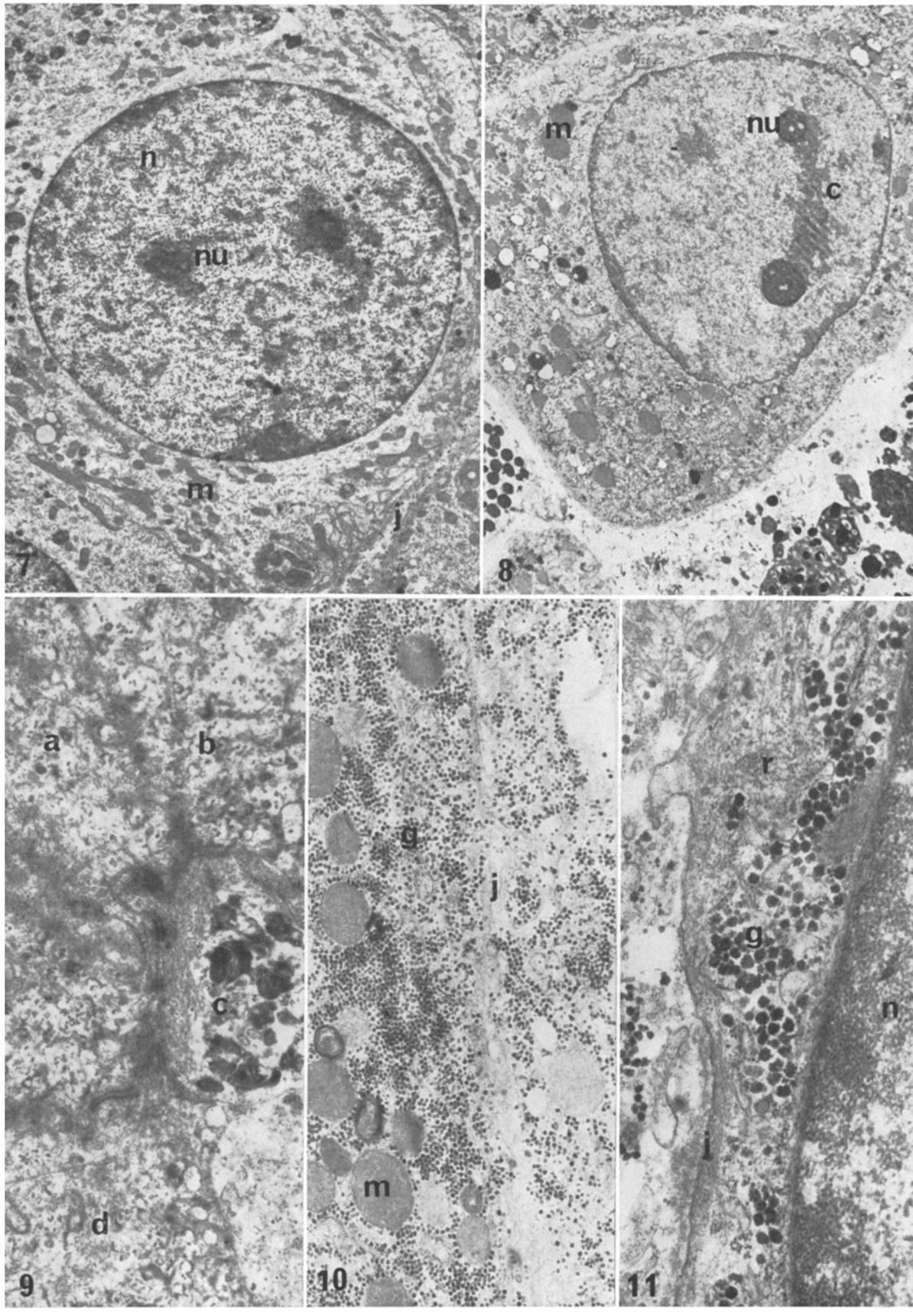
Fig. 2. Kidney, day 21, showing a glomerulus (g). ×150.

Fig. 3. Liver, day 28, showing retention of normal structure, hepatocytes (h) and pigment cells (p). ×150.

Fig. 4. Pancreas, day 21, showing nucleus (n), nucleolus (nu) and zymogen granules (z). ×2,100.

Fig. 5. Pancreas, day 21, showing endoplasmic reticulum (e), zymogen granules (z) and cell junction (j). ×10,500.

Fig. 6. Kidney, day 28, showing a nucleus (n), cell junction (j), and cytoplasm containing many mitochondria (m) and part of the Golgi complex (G). ×23,00.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The class *Amphibia* includes species with widely different cell sizes and DNA contents, including *X. l. laevis* with 6.3 pg DNA per 2C nucleus[89] and *A. means*, which has 168 pg DNA per 2C nucleus[90] – nearly 30 times the amount in human cells. The larger cells are ideal for studies on histology, histochemistry, mitotic and labelling incidence, and electron microscopy. Amphibians also tend to have relatively few, large chromosomes, and long-term diploid cell cultures are more readily obtainable than with mammalian cells[91]. Since amphibians are poikilothermic, their physiological and cellular processes are greatly influenced by temperature, which has profound effects on the rates of growth, development and cell division, and on levels of metabolic activity and the immune response. For instance, skin allografts in *X. l. laevis* survived 10 times as long at 10 °C as at 25 °C[92], and *Xenopus* did not produce antibodies against injected antigens when kept at 10 °C[93].

2. *Retention of normal structure and function in organ cultures of amphibian tissues.* Much of the published work on amphibian organ culture has concerned what might be called 'amphibian problems', such as the role of hormones and changes in enzymes during the fundamental changes that occur at metamorphosis, though much of the information obtained is of general importance. However, it has recently become clear that fragments of many organs from adults of a variety of species will survive for long periods in vitro. The significance of this finding is that present organ culture techniques have largely proved inadequate for culturing normal adult mammalian liver, kidney, pancreas and spleen for more than a few days[94–96]. In one of the more successful studies, CAMPBELL and HALES[94] cultured small pieces (1.5×1.5×0.5 mm) of mature rat liver in a gas phase containing 5% CO_2. After 6 days in vitro a zone of viable cells remained around the periphery of the fragments, and the thickness of this zone depended on the oxygen tension; in 95% O_2 the zone was 0.3 mm thick. The glycogen content of fragments fell rapidly after explantation, and there was no detectable glycogen after 2 days in vitro. For comparison, we will briefly describe our own experiments with 3 mm cubes from *A. means* pancreas, liver and kidney, which were cultured for up to 35 days at 25 °C, using methods described elsewhere[42]. After 21 days in culture, the pancreas fragments appeared histologically normal (Figure 1) and there was a marked increase in proliferation in the acinar cells. A low-power survey in the electron microscope of day 0 pancreas showed acinar cells with large nuclei, masses of endoplasmic reticulum and many zymogen granules. At higher mangnification it could be seen that this endoplasmic reticulum was studded with ribosomes and arranged in concentric circles. After 21 and 28 days in culture, the acinar cells still appeared normal and contained large amounts of endoplasmic reticulum and zymogen granules (Figures 4 and 5), though there may have been fewer granules than in day 0 cells. The mitotic incidence of kidney cells increased during the 28-day culture period, but although preservation at the periphery of fragments was excellent (Figure 2), some necrosis occurred at the centres of larger fragments[42]. Electron microscope preparations of day 28 kidney fragments showed that many cells were in good condition (Figure 7). They contained large numbers of mitochondria (Figure 6) and retained normal cell-cell contacts (Figure 9). Liver fragments were well preserved for the entire 35-day culture period (Figure 3). Electron microscope preparations showed that pigment cell complexes and hepatocytes retained their integrity (Figure 8), and on day 35 the hepatocytes still contained large amounts of glycogen and retained normal cell-cell contacts (Figures 10 and 11).

It has already been mentioned that pancreas fragments from *A. means*, *T. c. carnifex* and *X. l. laevis* continued to produce amylase in vitro. Preliminary electrophoretic studies[97] have indicated that normal tissue-specific enzyme patterns are retained in vitro. No changes were detected in lactate dehydrogenase (LDH) or esterase patterns of *A. means* pancreas, lung, spleen, liver and kidney during 35 days in culture. No changes were detected in LDH, malate dehydrogenase (MDH), acid phosphatase, esterase or glucose-6-phosphate dehydrogenase patterns in *X. l. laevis* kidney, liver, spleen, pancreas and ovary cultures during a 30 day culture period. No changes were

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Fig. 7. Kidney, day 28, showing nuclei (n), nuclei (nu), cytoplasm containing many mitochondria (m) and cell junctions (j). ×2,700.

Fig. 8. Liver, day 35, showing a hepatocyte containing mitochondria (m), a large amount of glycogen, and a nucleus containing two nucleoli (nu) with an apparent chromatin bridge (c) between them. ×2,900.

Fig. 9. Kidney, day 28, showing desmosomes at the junctions of 4 cells (a, b, c, d). ×19,000.

Fig. 10. Liver, day 35, showing hepatocytes containg mitochondria (m), large numbers of glycogen granules (g), and a cell junction (j). ×12,600.

Fig. 11. Liver, day 35, showing hepatocytes containing ribosomes (r), glycogen granules (g), a nucleus (n), and a cell junction (j). ×25,500.

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detected in LDH, MDH or alcohol dehydrogenase patterns in *T. c. carnifex* kidney, spleen and liver during 13 days in vitro. No quantitative estimations of these enzymes have yet been carried out, and we do not know whether synthesis of these enzymes continued in vitro, but the main point is that no new bands appeared. In comparison, enzyme patterns in primary monolayer cell cultures from *X. l. laevis* kidney, liver and muscle differed from in vivo patterns within a few days of being established.

3. *Reasons for the comparative success of amphibian organ culture.* One of the reasons why amphibian organ fragments culture well is that they are maintained at comparatively low temperatures where the solubilities of oxygen and carbon dioxide are increased and thermal inactivation of medium constituents is decreased[4]. This cannot be the only reason, since the success of long-term organ culture varies between species. We have found, in general, that urodele tissues culture better than anuran tissues, and fragments of organs from species with larger cells culture better than fragments from species with smaller cells[98]. A further reason for prolonged viability is that tissues may have variable respiration rates, depending on the oxygen tension. Cells in *A. means* fragments, for example, may be able to reduce their respiration rates as the oxygen tension falls in the centres of fragments. There seems to be an inverse relationship between tissue respiration rate and success of organ culture. *A. means* tissues, with the largest cells and lowest respiration rates, survived better than *X. l. laevis* tissues, with the smallest cells and the highest respiration rates. *T. c. carnifex* and *S. mexicanum* tissues, with intermediate cell sizes and respiration rates, survived in long-term organ culture better than *X. l. laevis* tissues, but not as well as *A. means* tissues.

Another contributing factor is the tolerance of amphibian tissues to variations in environmental conditions. Adult *A. means* liver and spleen fragments cultured well in media with 30–90% Leibovitz L-15 (125–305 mosmol/kg), and liver, spleen, lung and kidney survived as well in media with 0.8% foetal calf serum as in media with 8% serum, though higher rates of cell proliferation occurred in kidney fragments cultured in media with higher L-15 and serum concentrations[42].

6. *Prospects for future research*

It is clear from the preceeding sections of this review that many interesting problems have been tackled with the use of amphibian organ cultures. Problems which we consider deserving of attention in future research include the following.

Since many amphibian tissues survive long enough to complete several cell cycles in vitro, organ cultures could be used for more detailed investigation of the effects of temperature, tissue extracts and differences in cell size and DNA content on the control of the cell cycle in conditions closer to the in vivo situation than those prevailing in cell monolayer cultures. ROTHSTEIN and his associates have looked at the events leading up to mitosis in lens epithelial cells, which are stimulated to proliferate in synchrony in vitro. However, it is not known why or how these cells, which do not normally proliferate in vivo, are activated. CHOPRA and SIMNETT[50] have evidence for a tissue-specific inhibitor acting in late G2 in pronephric cells. REDDAN and ROTHSTEIN[60] have some data on the effects of temperature on the cell cycle times of lens epithelial cells at various temperatures. CHIBON[99], using *P. waltlii* larvae, has found that although the cell cycle times of various tissues decreased as the temperature was raised, S, G2 and M phases were shortened, whilst G1 became longer. As the temperature was lowered, S, G2, M and the total cycle time became longer, G1 became shorter. It would be interesting to know whether this is a general phenomenon in poikilotherms, possibly serving as a mechanism for overcoming different effects of changes in environmental temperature on cell production, function and loss.

Since primary immune responses can be induced in *X. l. laevis* spleen in culture, it would be interesting to examine the effect of temperature on the proliferation and maturation of antibody-secreting cells, which are influenced by temperature in vivo[100].

It would be extremely useful to have a completely defined medium for amphibian organ culture, since a survey of the literature reinforces the feeling that we have little systematic information on what is essential for the maintenance of tissues. Long-term cell and organ culture usually requires serum, though SOOY and MEZGER-FREED[101] found that a macromolecular fraction of foetal calf serum, together with added purines, supported the growth of a number of amphibian cell lines. More work of this nature is essential.

Since fragments of visceral organs from adults can be successfully cultured, such cultures could and should be used to study the control of major metabolic processes and the action of hormones. The effects of thyroxine in inducing urea cycle enzymes in liver have already been studied, and it is interesting to note that liver cubes were used in these experiments, because thyroxine had no effect on protein sythesis or specific enzymes in liver cell suspensions[39]. Further systems for study are the control of glucose storage in the liver,

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and the control of exocrine and endocrine activities in pancreas cultures.

Work on lens regeneration has shown that neural retina is vital for lens regeneration from the iris. Extrinsic factors have also been cited as important in limb regeneration, and in view of the ease of testing such factors in vitro, it is surprising that so little work has been done on amphibian limb regenerates in organ culture.

Heterotypic interactions between epithelial and mesenchymal tissues are essential for the normal development of many organs (e.g. kidney [102]). One of the most important findings from research on induction in vitro was that there are mutual control systems between interacting tissues [103]. TARIN [104] has stressed the importance of heterotypic interactions in the maintenance of normal structure and function in the adult and their possible significance in carcinogenesis. We consider that amphibian organ cultures are of great potential importance in seeking solutions to these problems [105, 106].

Note added in proof. A number of articles have appeared since this review was written. CLEMENS, LOFTHOUSE and TATA [107] have repeated WALLACE and JARED'S [40] experiments on *X. l. laevis* liver, using serum-free, HEPES-buffered medium 199 with labelled amino acids. They found that liver explants from oestrogen-treated males secreted 4 times the amount of labelled protein as explants from untreated males, while there was no increase in the amount of radioactivity in tissue proteins over a 4-day period. Inhibitors of RNA synthesis inhibited the secretion of protein after 2–3 days. BALINSKY, COETZER and MATTHEYSE [108] cultured adult *X. l. laevis* liver cubes for up to 8 days in medium 199, and looked at the uptake of ^{3}H-leucine into carbamyl phosphate synthetase in animals which had been kept in normal or hypertonic saline. Animals kept in hypertonic saline excreted a higher proportion of their nitrogenous waste in the form of urea and had higher levels of carbamyl phosphate synthetase. Liver explants from such animals incorporated more ^{3}H-leucine into enzyme and experiments with puromycin showed that, unlike *R. catesbeiana* [65], no non-immunoprecipitable enzyme precursor was involved. MAHDAVI and CRIPPA [109] incubated ovaries from *X. l. laevis* tadpoles for 48 h in Leibovitz L-15 medium containing 10% foetal calf serum, ^{3}H-uridine and ^{14}C-thymidine to label the RNA-ribosomal DNA complex. HARPER and GROSS [110] have shown that *R. catesbeiana* tadpole tissues which produce collagenase in vitro, first secrete an inactive zymogen and later an activator, which converts the zymogen to active enzyme.

Résumé. Les auteurs passent en revue les problèmes étudiés et les méthodes utilisées jusqu'à ce jour pour la préparation des tissus des amphibiens en culture organotypiques. Ils envisagent en outre les possibilités qui s'offrent aux recherches futures.

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[106] *Appendix.* Full names of species listed in Tables I and II but not given in full in the text: *Bufo bufo bufo, Bufo marinus, Discoglossus picta, Hyla arborea savignji, Pleurodeles waltlii, Rana clamitans, Siredon mexicanum, Taricha torosa, Triturus cristatus.*
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SPECIALIA

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Arctigenin-4′-β-Gentiobioside from *Trachelospermum asiaticum* var. intermedium

In addition to the reported isolation of 4 lignan glucosides, arctiin, matairesinoside, tracheloside and nortracheloside from the stems of *Trachelospermum asiaticum* Nakai var. intermedium Nakai (Apocynaceae) [1–4], we now found a new lignan glycoside, arctigenin-4′-β-gentiobioside (I) the first example of a naturally occurring glucosyl glucoside of the lignan series.

The glycoside (I), a colorless crystalline powder, mp 174–176°, λ_{max}^{EtOH} nm (log ε) 230 (4.20), 280 (3.79), ν_{max}^{KBr} cm^{-1} 3400 (br. OH), 1770 (CO), 1595, 1515 (aromatic, C=C), $[\alpha]_D^{26}$ −57.2 (c = 1.0 in H_2O), Anal. Calcd. for $C_{33}H_{44}O_{16}\cdot H_2O$: C 55.46, H 6.49; Found: C 55.63, H 6.55, is obtained in 0.0004% yield from the chloroform-methanol (2:1 V/V) extractive of the residue after the extraction of four other lignan glucosides and gave heptaacetate, colorless needles, mp 183–184°, λ_{max}^{EtOH} nm (log ε) 229 (4.22), 279 (3.81), ν_{max}^{KBr} cm^{-1} 1760 (CO), 1595, 1515 (aromatic C=C), $[\alpha]_D^{21}$ −46.7 (c = 1.168 in $CHCl_3$), Anal. Calcd. for $C_{47}H_{58}O_{23}$: