MAINTENANCE OF GONADS OF FROG LARVAE IN ORGAN CULTURES

CHARLES L. FOOTE AND FLORENCE M. FOOTE
Southern Illinois University, Carbondale

INTRODUCTION

A number of media have been used for the cultivation *in vitro* of amphibian tissues and organs (Wilde, 1948; Preston, 1949; Boss, 1954; Foote and Foote, 1957). Such media usually contain blood serum or plasma and embryo extracts from mammalian or avian sources. These culture media are adequate for the maintenance of tissues and organs, and they usually provide the proper nutritional elements for growth and proliferation of cells. In attempts to grow amphibian gonads for the study of problems of sex differentiation, it would seem that tissue extracts from amphibian sources might produce better growth of amphibian organs. Preliminary results are presented here in which gonads were placed in cultures containing tissue extracts of larval amphibians and compared with organs grown on media containing chick embryo extract.

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MATERIALS AND METHODS

Second-year larvae of *Rana clamitans* were collected locally. Twenty-four hours before gonads were to be cultured the tadpoles were placed in a solution of 1% sulfadiazine in aerated tap water to which had been added 10 to 20 gamma/ml. of streptomycin sulfate (Wilde, 1948). Gonads were carefully removed, under aseptic conditions, from animals which had been anaesthetized with M. S. 222. Mesentery and fat bodies were dissected away and one gonad of each pair was placed in sterile Holtfreter's or Tyrode's solution for about two hours before it was put into culture. The other gonad of the pair was preserved as a control.

The composition of the two media used in this study was as follows:

MEDIUM I

Amphibian tissue extract (50%)	2 parts	16.7%
1% agar	6 parts	50.0%
Glass-distilled water	3 parts	25.0%
Streptomycin solution, 5 gamma/ml. in water	1 part	8.3%

MEDIUM II

Chick embryo extract (33%)	4 parts	20.0%
1% agar	13 parts	65.0%
Glass-distilled water	2 parts	10.0%
Penicillin, 1% solution in water	1 part	5.0%

Amphibian tissue extract was prepared from 20 *Rana clamitans* larvae from which the intestinal tract had been removed. The larvae were placed in a Waring Blendor and macerated for approximately five minutes. To the macerated tissue was added an equal volume of full strength Holtfreter's solution. When this mixture was homogeneous it was placed in 50 ml. tubes and centrifuged for 10 minutes at 3500 r.p.m. The supernatant fluid was then filtered through a Seitz bacterial filter (0.1 μ) and the filtrate placed in sterile vaccine bottles and refrigerated at 2 to 6° C.

The chick embryo extract was prepared from chick embryos which had been incubated for 11 days. Ten embryos were macerated in a Waring Blendor for 5 minutes, the resulting tissue was placed in 50 ml. centrifuge tubes and diluted to the following proportions: 2 parts embryonic tissue, 1 part Tyrode's solution with 1% glucose added, and 3 parts glass-distilled water. After centrifugation at 3500 r.p.m. the embryo extract was frozen at -4° C. before use.

In preparing final media an attempt was made to have the concentrations as nearly isotonic as possible for amphibian tissues. Watch glass cultures were made according to the method of Fell and Robison (1929) and gonads were placed on solid media. Cultures on Medium I consisted of 14 testes and 10 ovaries and on Medium II 8 testes and 22 ovaries. The maximum period of time for cultivation of these organs was 29 days.

Cultures were kept in a constant temperature cabinet at 24 to 26° C. and maintained at pH 7.2 to 7.4. Organs were transferred to fresh media each fifth day. At the end of the culturing period organs were fixed in Zenker's solution, sectioned at seven microns, and stained in Harris' hematoxylin and chromotrope 2-R.

Results

As criteria for determining which medium was most nearly ideal for growth and maintenance of amphibian testes and ovaries, the following conditions were observed: proliferation and migration of cells, maintenance of organs without growth, and degree of necrosis (Table 1).

Ovaries appeared to grow and retain their characteristic form better than did testes, although auxocytes in the ovaries showed progressive degeneration beginning during the first week of the culture period (Fig. 1, A, E). This condition is usually found in ovaries where insufficient amounts of gonad-stimulating or sex hormones are present. Other cells of the organ did not show much degeneration. Both types of gonads seemed to grow better on media containing chick embryo extract (Fig. 1), with most of the cellular growth occurring among the non-germinal cells. The term non-germinal is employed to designate follicle cells, granulosa, theca, stroma, and peritoneum. Occasionally, immature germ cells or even auxocytes were carried with the non-germinal cells as they migrated (Fig. 1, C); however, the greatest cell proliferation occurred among the granulosa cells (Fig. 1, B). This was particularly noticeable in ovaries where degeneration of auxocytes occurred since,

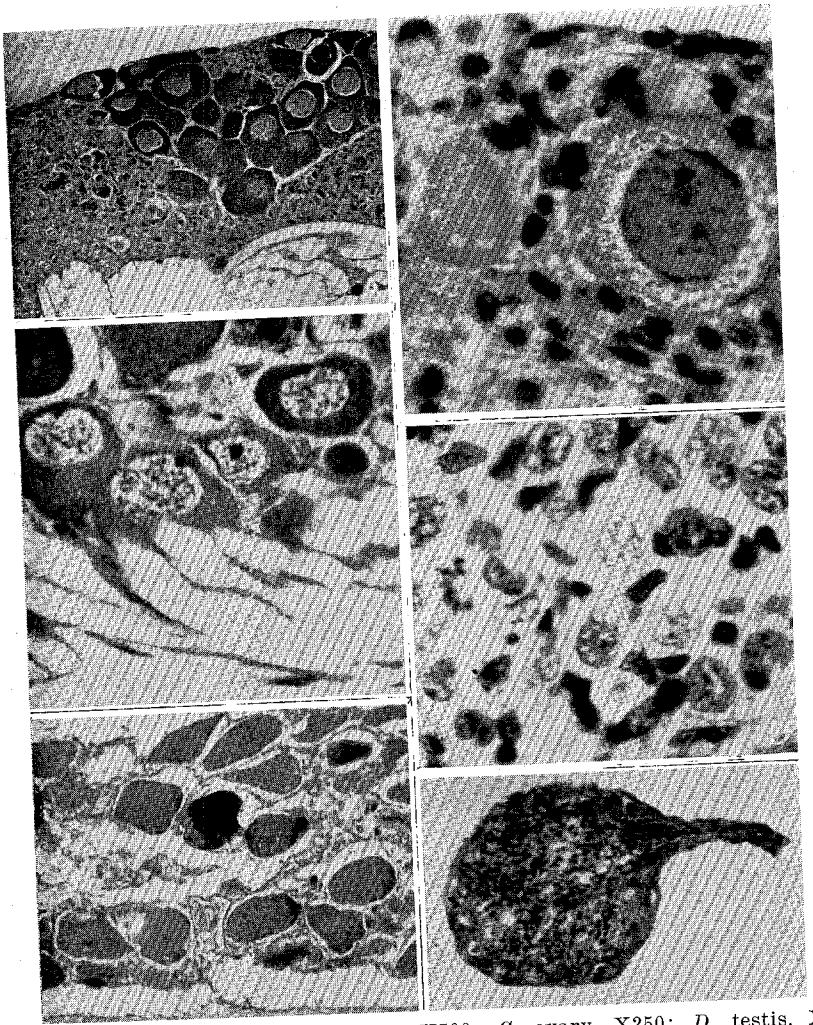

FIG. 1.—*A*, ovary, X60; *B*, ovary, X500; *C*, ovary, X250; *D*, testis, X500; *E*, ovary, X120; *F*, testis, X120. *A*, *B*, *C*, *D*, on Medium I; *E* and *F*, on Medium II. Culture time, 28 days.

as the large cells decreased in size, the granulosa cells filled in the space in the follicle. There were mitotic figures, not only in non-germinal cells, but in germ cells as well (Fig. 1, D).

No mitoses were observed in organs grown on amphibian tissue extract, and there were only isolated cases in which a few non-germinal cells had migrated from the explant. In some instances both ovaries and testes appeared to be well maintained with cells in their characteristic form, although in others partial or complete necrosis occurred (Fig. 1, E, F).

TABLE 1.—Numbers of Gonads Showing the Various Conditions Described.

	Amphibian tissue extract and agar (medium I)		Chick embryo extract and agar (medium II)	
	ovary	testis	ovary	testis
Growth and proliferation...	0	0	19	2
Maintenance without growth	7	5	0	3
Necrosis.....	3	9	3	3

DISCUSSION

From the results obtained in the present experiments it appears that amphibian gonads grow better on a medium containing chick embryo extract than on amphibian tissue extract. Had amphibian plasma or even chick plasma been added to the amphibian tissue extract better results might have been obtained. However, Preston (1949) and Boss (1954) reported that media containing cock plasma and chick embryo extract served adequately for growth of a variety of amphibian tissues.

For purposes of studying sex differentiation in the amphibian gonad it appears that the best medium would be chick embryo extract and agar. On this medium there is little cellular migration, with few changes in cell form. There is cell multiplication among non-germinal cells and germ cells. This medium appears to be adequate.

While some necrosis occurred on all types of media, ovaries were better maintained than testes. This may be due to the fact that the ovaries with a larger surface area exposed to the medium absorbed greater amounts of nutritive material than testes with a much smaller surface area. It may also be taken into account that some of the deutoplasm from degenerating auxocytes may have been utilized by other cells, but most of the nutriment for the organs came from the medium.

SUMMARY AND CONLUSIONS

1. Ovaries and testes of second-year *Rana clamitans* larvae grow well in organ cultures.
2. A medium containing chick embryo extract causes some cellular migration, but germ cells as well as non-germinal cells multiply in both ovaries and testes.
3. There is little growth of organs on a medium of amphibian tissue extract and agar, although 50% of the organs can be maintained on this medium without apparent cellular change.

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