ORGANS OF THE LARVAL AXOLOTL (*SIREDON MEXICANUM*) GROWN SINGLY OR IN COMBINATION *IN VITRO*

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

ABSTRACT.—Organs of the larval axolotl were grown either singly or in various combinations in watch glass cultures for 21 days. Testis, mesonephros, brain, spleen, pancreas, heart, ovary and liver, when grown alone, were each well maintained except for the liver. Combinations of ovary and testis resulted in suppression of germinal cells of the ovary. Testes, when grown with mesonephros, showed lumina in some of the seminiferous tubules. Testes and other organs, when paired, were similar to the same organs grown alone. Ovaries, paired with mesonephros, spleen, or brain, had fewer degenerating oocytes and proliferation of young germ cells and non-germinal elements. Ovarian tissue was destroyed by pancreas, and when paired with liver, neither organ was well maintained.

The *in vitro* cultivation of tissues and organs from cold-blooded vertebrates has been attempted by several investigators. Wilde (1950, 1955) and Wolf and his co-workers (1957, 1960) have maintained cells and tissues of fishes and amphibians. Foote and Foote (1958, 1959, 1961, 1962) have used a number of amphibian species to study the development of the reproductive system *in vitro*. The present paper is a report of efforts to cultivate tissues and organs from the axolotl (*Siredon mexicanum*), and to determine whether there is any beneficial result from growing two different organs together.

MATERIALS AND METHODS

Larval stages of the axolotl, 4 to 5 months of age, each with a total body length of 10-12 cm., were used. These larvae were placed for 24 hours in large finger bowls containing solutions of sulfadiazine and streptomycin in an attempt to make them bacteriologically sterile. To remove the organs for culturing, the animals were decapitated and pinned out in sterile wax-filled dissecting pans. Care was taken not to rupture the gut since the antibiotics were not ingested in observable amounts by the larvae. Organs were dissected, cut into small pieces for explanting into culture, and placed in Tyrode's solution diluted 2:1 with double distilled water. Culture methods employed were those of Wolff and Haffen (1952), modified for amphibian tissues. The culture medium was as follows:

Tyrode's solution (2:1)	3 parts
Chick embryo extract (EE50)	3 parts
Distilled water	4 parts
Agar-agar (1%)	5 parts
Penicillin & Mycostatin	

These components were put into sterile embryologic watch glasses and allowed to solidify. The explants were then placed on the solid substrate, usually 5 or 6 explants arranged in a circular pattern for culture of single organ pieces. In the case of the "sandwich" preparations, the ovary was cut transversely into a number of sections and small pieces of other organs were placed inside the ovarial cavity before putting the combination on the medium.

The two organs were thus maintained in intimate contact during the culture period. Other preparations of pairs were placed side by side in contact on the culture medium. A cover glass was placed over the watch glass and sealed with melted paraffin, making sure the seal was airtight. Cultures were maintained at approximately 21-24 degrees C. Dishes were opened and explants transferred to fresh media each fifth day. After a culture period of 10 to 21 days, explants were removed from culture and fixed in Bouin's solution. They were then sectioned by the paraffin method and stained in hematoxylin and eosin.

Organs placed in culture were testes, ovaries, heart, liver, mesonephros, pancreas, spleen and brain and combinations of these in pairs. Table I shows the number of explants and the time in culture.

Results from Tissues Grown Alone in Culture

Testis-Control. The general appearance of the control testis is that of very closely packed cells with no spaces except for an occasional blood vessel. The beginning of the formation of seminiferous tubules is indicated by the fact that the germ cells occur in groups of 2 or 3, surrounded by follicle cells, but with no apparent lumina. The germ cells are in pre-miotic stages (spermatogonia or primary spermatocytes); their nuclei are spherical, unlobulated, and they stain less darkly than those of the follicle cells. The cytoplasm of the germ cells is clear. Explants were taken from both the center and periphery of the gonad and as a result some do not have any part of the tunica albuginia.

Testis-Cultured. In explants cultured for only 3 to 4 days, the germ cells and seminiferous tubules show no marked changes from controls. There is a somewhat less compact appearance due to a looser arrangement of non-germinal elements. After 10 days in culture a continued proliferation of non-germinal elements takes place, resulting in a less compact appearance. Germ cells remain as in the controls, except that lumina appear in a few of the seminiferous tubules. After 21 days in culture the non-germinal cells have increased greatly in number and may make up as much as half of the explant. The germ cells are fairly well maintained, but the demarcation between nucleus and cytoplasm is less distinct and the chromatin stains less densely than in the controls (Pl. I, UR).

Ovary-Control. The cortex of the ovary is somewhat less compact than the testis. It is surrounded by the theca externa. There is a large ovarial cavity. At the periphery of the ovary there are nests of 5 or 6 gonial cells with large lobulated nuclei and with very little cytoplasm. At this stage of development the greater part of the cortex is composed of auxocytes, i.e. oocytes with little deposition of yolk. Their nuclei are large and spherical, containing many nucleoli, but with no indication of chromosomes. The auxocytes have few follicle cells apparent and are supported primarily by the cells of the theca interna. Ovarial explants were cut in such a way that each piece had a portion of the theca externa on one side.

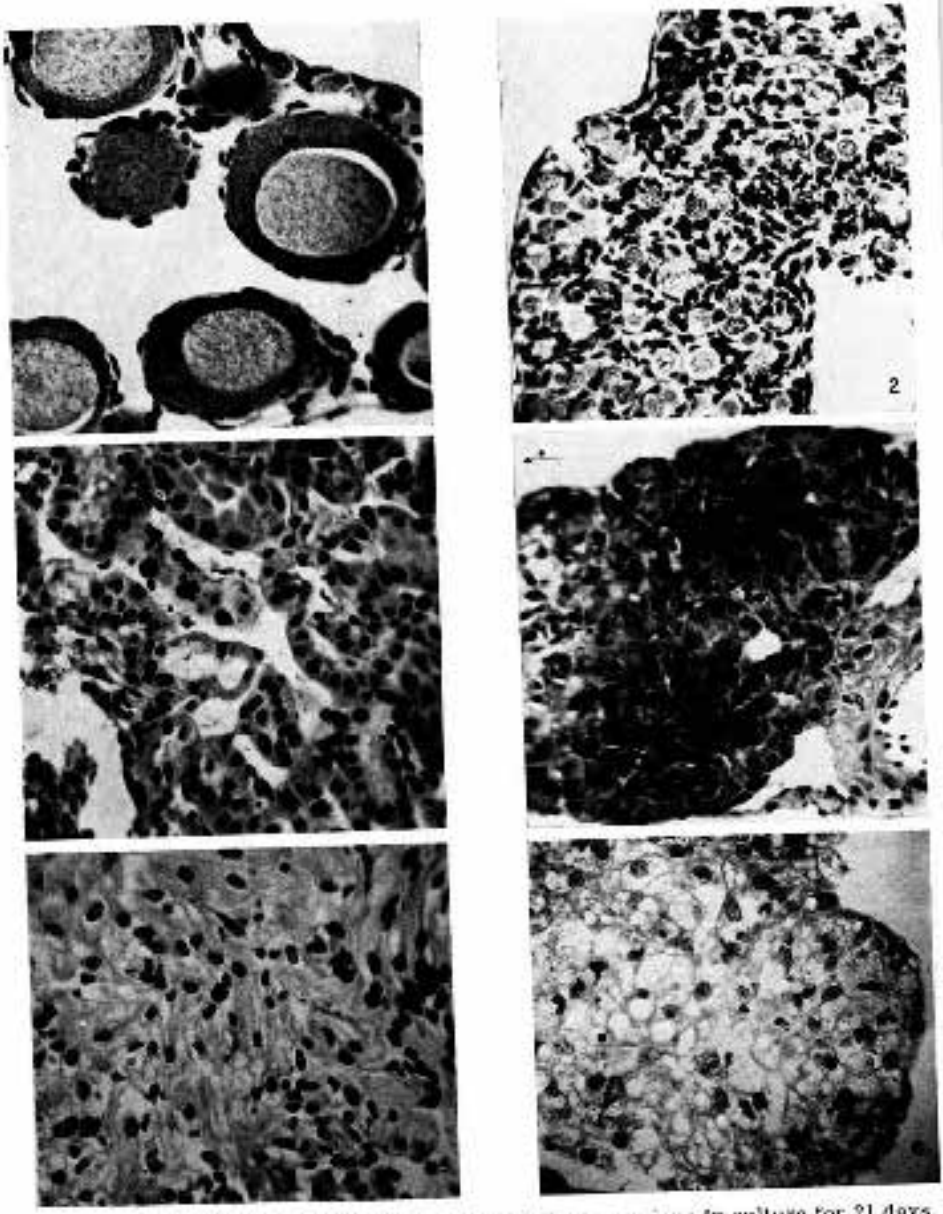

PLATE I. Sections of organs of axoloti, grown alone in culture for 21 days. x200. Upper left (UL), ovary; upper right (UR), testis; middle left (ML), mesonephros; middle right (MR), pancreas; lower left (LL), heart; and lower right (LR), liver.

Ovary-Cultured. Ovaries in culture for 21 days show the same general structure and types of cells as do the controls (Pl. I, UL). There is some indication of degeneration of cells, especially large ovocytes, but there are no large necrotic areas. Auxocytes have large nuclei showing dark staining chromatin. Oogonia appear to be in good condition and are located near the periphery of the explant. They have lobed nuclei and distinct cytoplasm. The main change from the controls appears to be in the non-germinal cells. These have proliferated somewhat from some areas of the explant and appear to have originated from stromal and thecal cells. Some sections of the ovary show groups of cells arranged near the periphery in such a manner as to form "bubbles." Many explants showed these "bubbles" while they were still in culture. In some explants the thecal membranes are very distinct and the auxocytes seem to have shrunken, making the cells of the thecal layers more conspicuous.

Heart-Control. The cardiac muscle fibers are distinct but show no striations in these preparations. Fibers form distinct bundles which lie in different planes and run in various directions. Anastomosing bundles can be observed. There are many nuclei of essentially ovoid form which stain darkly. An occasional mitotic figure is seen.

Heart-Cultured. Explants of heart continue to beat while in culture. Most explants show pulsation up to ten days, but after that time there is a decrease in movement. In stained and sectioned material the fibers and nuclei do not look too healthy, even in those explants which showed contractions during the culture period. In these explants there are always outgrowths of cells. These do not proliferate as individual cells but extend away from the explant as more or less solid sheets of cells. The area of outgrowth has the same form as the heart muscle itself, but always stains with a basic stain whereas the main body of the explant stains with acid stain. It appears that the typical cytoplasm of the muscle fiber has not yet differentiated in the outgrowth from the explant. The heart explants seem to be somewhat reduced in size after being in culture for 21 days and the fibers are more closely massed together (Pl. I, LL).

Liver-Control. The liver is a reticular gland composed of many cells which in cross-section have 3, 4 or 5 sides. While the cells are roughly square in outline, they are very irregular and vary considerably in size. The liver is not divided into lobes, as it is in man and higher vertebrates. Each cell has a rounded nucleus surrounded by a large area of cytoplasm which contains dark staining granules. The outline of each cell is very distinct due to the dark staining reaction of the material between the cells. The outermost cells of the liver are closely packed and have very little cytoplasm and relatively large nuclei; in this area the cytoplasm and cellular outline, are not distinct due to the multitude of nuclei. There are occasional mitotic figures. Pigment cells and large blood and lymph vessels are interspersed in the tissues of the liver.

Liver-Cultured. The most marked

change in liver explants following a period in culture is a decrease in size of the explant. This is due for the most part to the loss of individual cells from the periphery of the explant while it is in culture. Apparently the cells do not adhere well to each other and a capsule around the explant is not formed. After 10 days of cultivation, cells retain their irregular form, each with a distinct nucleus, but the cytoplasm becomes filled with small vacuoles. After 21 days in culture, the cytoplasmic vacuoles have increased in size so that the explants have the appearance of a reticulum with numerous nuclei, many of which seem to be necrotic (Pl. I, LR).

Mesonephros-Control. The kidneys are elongated and composed of mesonephric tubules with few glomeruli and large Bowman's capsules. The tubules vary in size as does the height of the epithelium in each tubule. Each cell has a distinct ovoid shaped nucleus. Some pigment cells are present especially in the areas of the blood vessels. In some sections the Mullerian and Wolffian ducts are present.

Mesonephros-Cultured. Mesonephric kidneys maintained in culture as long as 21 days show no marked changes from control organs (Pl. I, ML). The tubules remain distinct with no apparent changes in cell form or structure. There are some tubules which are distended, due apparently to accumulated fluids. Some tubules contain material which may have been secreted from the cells or perhaps taken from the medium through the nephrostomes. The gonaducts seem to be well maintained in culture and retain their normal appearance.

Pancreas-Control. This organ has a lobulated appearance, but it is compact and individual alveoli are not distinct. The cell form varies from cuboidal to columnar. The nuclei are large and rounded and centro-acinar cells are present. Many cells have secretory granules in the apical parts of the cells. The islet cells can be identified.

Pancreas-Cultured. This organ seems to be in very good condition after 3 weeks in culture. In some areas of the explant the alveoli are much more distinct than they are in the controls. This may be due to a separation of one alveolus from another after the organs were dissected for explants or it may possibly be due to a loss of connective tissue while the organs were in culture. Some explants look as compact as in the control material (Pl. I, MR) Secretory granules are very conspicuous in pancreatic cells, but less abundant than in the controls. Some islet cells are present.

Spleen-Control. The spleen is a rounded organ, surrounded by a connective tissue capsule. There are no trabeculae, nor are there definite groups of white cells as seen in the spleen of higher vertebrates. Leukocytes and erythrocytes seem to be distributed throughout the organ, in about equal numbers. Mitotic figures are very common. The reticular cells are large and form a supporting network for the blood cells.

Spleen-Cultured. The form of the spleen is very similar to that control, and the explants show little decrease in size. The number of

leukocytes is approximately equal to that of the controls, but few erythrocytes remain in the explants. The reticulum is much more distinct after culturing and the reticulum makes up more than half the bulk of the explant. Some mitotic figures were observed in cultured material.

Brain-Control. The part of the brain used in this study was the diencephalon. Areas of grey and white matter are very distinct. The grey matter, nearer the brain cavity, contains at least two distinct types of nerve cells, whereas the white matter is composed primarily of fibers and supporting tissues. In some sections the choroid plexus is present.

Brain-Cultured. Cultured explants show the same general plan as controls, with distinct areas of grey and white matter. In the grey matter the two types of nerve cells can be identified easily. A few vacuoles have formed in the supporting tissue of the grey matter and some of the nerve cells are more clumped together. The nerve cells of the explants, however, seem to be in excellent condition. Some mitotic figures were observed in the grey matter.

Results from Tissues Grown in Combinations in Culture

Testis and Ovary Cultured. Explants of both testis and ovary have a very loose structure and give the appearance of a network of connective tissue supporting the germ cells. There has been some proliferation of non-germinal cells from both organs and these have grown in contact with each other. Whereas the germ cells of the testis look essentially normal, the explants of the ovary contain few germ cells, auxocytes or oogonia. Only about 10% of the usual number of ovarial germ cells remain in the explants (Pl. II, UL).

Testis and Heart-Cultured. Explants of testis and heart are grown together and cells from the heart have invaded the gonad. Neither organ, however, appears to be in good condition. The explants of the testis lack the characteristic organization and few germ cells are present. Fibers of cardiac muscle are distinct, but no striations are visible.

Testis and Liver-Cultured. Explants of testis and liver are very closely attached. Germ cells of the testis look healthy with normal appearing follicle cells. The testis is very compact. More connective tissue is present in the testis and the testis portion of the explant is encapsulated, whereas the liver portion is not. Liver explants appear very similar to those cultured alone, with loss of typical cell structure and organization.

Testis and Mesonephros Cultured. Testis in combination with mesonephros looks essentially normal (Pl. II, UR). Explants are compact and with less connective tissue between the seminiferous tubules than when grown with liver. In some instances the cells from the mesonephric tubules have intermingled with the germ cells of the testis. In some explants of mesonephros grown with testis, the Wolffian ducts appear to be more dilated than those of kidney grown alone.

Testis and Pancreas-Cultured. The pattern of testis and pancreas grown together differs from that of

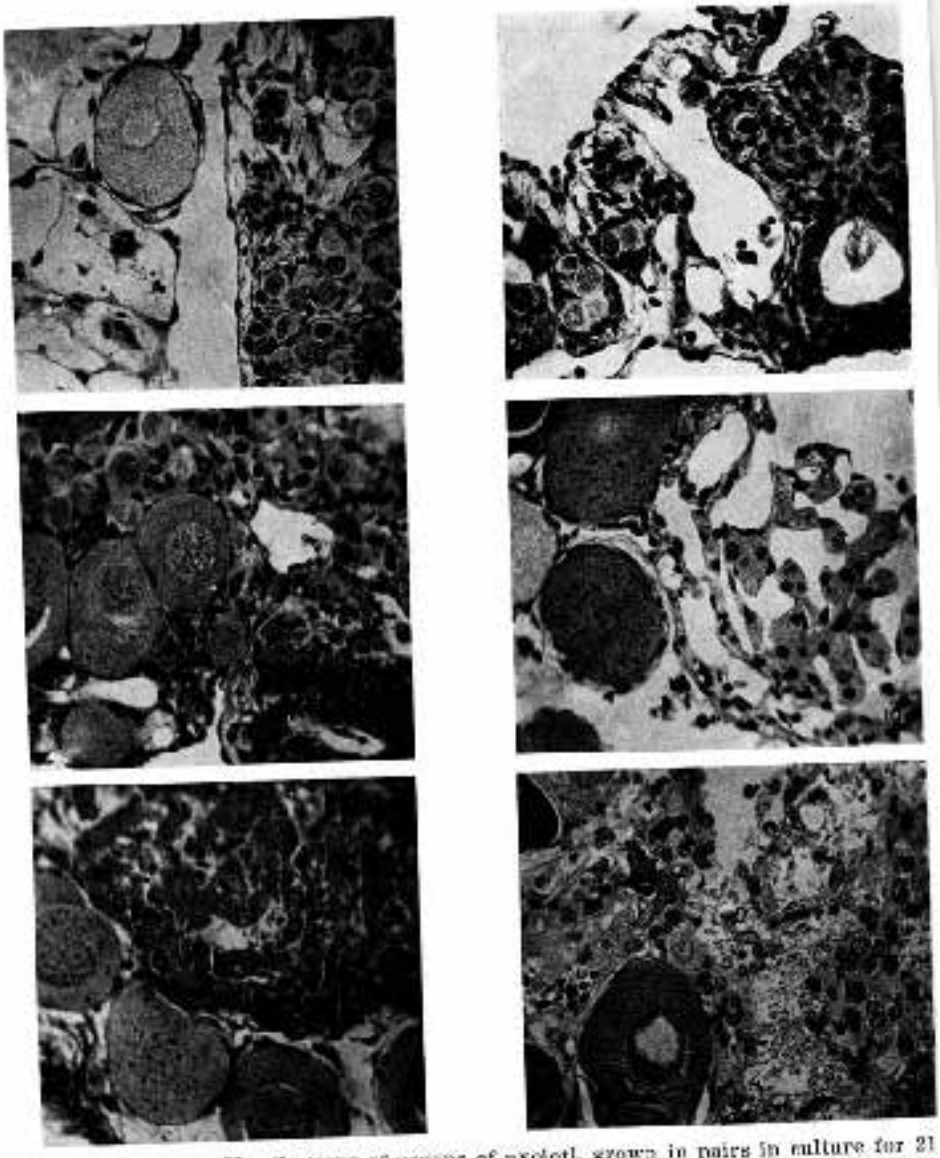

PLATE II.—Sections of organs of axolotl, grown in pairs in culture for 21 days. ×300. UL, ovary and testis; UR, testis and mesonephros; ML, ovary and mesonephros; MR, ovary and pancreas; LL, ovary and heart; and LR, ovary and liver.

the testis and liver and of the testis and mesonephros combinations. The tissue of the testis is much less compact, but there are spaces between the seminiferous tubules. Such areas in explants of testis grown alone con-

tain connective tissue. The germ cells are as well maintained as when testis is cultured alone. The pancreas explants look healthy with distinct alveoli. Some cells from the pancreas explants have grown around the testis explants to form a capsule.

Ovary and Heart-Cultured. In this combination there is an intermingling of cells from the two organs whether they are placed side by side or whether the heart explant is inserted into the ovarial cavity. The heart appears characteristic for cultured material. The ovaries as well maintained with only a few degenerating auxocytes. There is little proliferation of non-germinal cells (Pl. II, LL).

Ovary and Liver-Cultured. These explants were placed side by side or explants of liver were inserted into the ovarial cavity. Neither explant appeared to be in good condition (Pl. II, LK). The ovary showed many degenerating auxocytes, although some younger germ cells were present. The non-germinal cells had proliferated extensively. In some cases, where the cells of the liver were in close contact with the ovary, these cells appeared to be better maintained than were liver cells alone.

Ovary and Mesonephros-Cultured. There are very few degenerating auxocytes in ovaries in combinations with mesonephros, and the ovaries look very much like uncultured controls (Pl. II, ML). The explants of mesonephros show distinct tubules, occasional glomeruli, and Wolffian and Mullerian ducts. Some tubules, as well as the Wolffian and Mullerian ducts, may be dilated. In some combinations the cells of the kidney explants surround the auxocytes. In instances where kidney explants are placed in the ovarial cavities, these cavities are much distended and germ cells are located in a thin band surrounding the cavity and kidney explant.

Ovary and Pancreas-Cultured. Pancreas explants look healthy and similar to explants grown alone. However the ovarian explants have been almost totally destroyed, apparently by enzymes from the pancreas. Many partially degenerated auxocytes are surrounded by cells from the pancreas. A few oogonia and oocytes can be identified (Pl. II, MK).

Ovary and Spleen-Cultured. The ovary appears to be in good condition and all types of cells can be identified. The reticular cells of the spleen have in some instances invaded the ovary and surrounded the germ cells, resulting in some degeneration of auxocytes. The white blood cells of the spleen appear to be in excellent condition and some are undergoing mitoses. Red cells are present, but often are irregular in form.

Ovary and Brain-Cultured. Both explants of ovary and diencephalon appear healthy. In some instances the ovary has a few degenerating auxocytes, but younger germ cells are present. There has been some proliferation of non-germinal cells. The brain tissue is the more actively growing one; it has invaded the ovarian explants. Many fibers have proliferated from brain to ovary, especially where the brain was inserted into the ovarial cavity.

Discussion

The maintenance *in vitro* of embryonic cells and tissues of amphibians is a routine procedure in many laboratories. The cultivation of organs from larvae or adults is less frequently and less successfully accomplished. No comparative study of the survival in culture of different organs of a single species of amphibians exists. Neither has the possible effect of one organ on another *in vitro* been investigated in amphibians as it has been in some birds and mammals.

Wolff (1954) has demonstrated that different organs of one species of bird or mammal will grow together with the connective tissue elements often becoming indistinguishably intermingled. The more specialized tissues, such as epithelium of the bronchi or bronchioles, maintain their independence. Similar results were obtained with the culture of combinations of organs of different vertebrate classes, chick or duck and mouse, for example (Wolff, 1954; Wolff & Weniger, 1954; Salzgeber, 1962).

Wolff and Wolff (1961, 1962) succeeded in growing cancer tissue, taken directly from a human patient, in association with chick mesonephros. These cancer tissues were maintained for 36-45 days, through several transfers, and continued to manifest the pattern associated with the particular type of original malignancy. Such organized tumors will not survive alone on the medium, although cell lines of malignant cells have been maintained for years.

There have been attempts to determine which factors from one organ in culture stimulate another organ. Lutz-Ostertag and Fargeix (1962) found that embryo chick Mullerian duct grew better in the presence of chick heart, and that the production of certain amino acids by the heart tissue enriched the medium. This was in accord with result of Pasicka et al. (1956), concerning the amino acid metabolism of cultivated chick heart fibroblasts.

Croisille (1958) used extracts of various embryonic chick organs as substitutes for complete embryo extract in the medium and found that liver extract and heart extract produced poor survival of the organs cultured. However, he obtained satisfactory results with extracts of mesonephros, lung, skin and intestine.

The present experiments indicate that in amphibian organs there is an advantage, in some instances, to growing organs in combinations. It was of particular interest to find some method of culture which would maintain the ovaries for long periods of time, permitting the various types of cells to differentiate. The hollow structure of the ovary presented an excellent situation for testing any stimulating effect which might be exerted by an associated organ. Ovaries in combination with mesonephros, heart, spleen, and brain appeared to be well maintained, with fewer degenerating cells than when grown alone. Mesonephros was the most satisfactory of the above organs. Neither pancreas nor liver promoted growth of the ovary in combination with it. The digestive enzymes of the pancreas may have destroyed the ovarian tissue. The liver has not proved to be an organ which is well

maintained in culture itself (see below). It has no apparent effect on the ovary. In the ovary-testis combinations the testis remains healthy but many of the ovarian germ cells degenerate. This may be considered an illustration of the 'free-martin' effect.

The amphibian liver grows less satisfactorily in culture than does the liver of chick (Wolff, 1954) or mouse (Salzgeber, 1962). The cells become vacuolated and after 21 days in culture many of the cell membranes have disappeared, leaving a syncytial arrangement of both pycnotic and fairly normal looking nuclei. The organ fragment is difficult to handle, breaking into pieces when transferred to fresh medium or during preparation for histological study. Many cells slough off from the surfaces, giving the explant a ragged appearance. No surrounding membrane forms, although if the explant contains a portion of the serosa that side of the explant will have a surface membrane. The serosa is not extended to the other surfaces of the explant. Since containment within some sort of membrane is essential for growth as an organ, this may be the explanation of the poor results with amphibian liver. The only instance in this experiment in which a piece of liver was in fairly good condition was one which was grown inside the ovarial cavity. It might be that the tissue of the ovary served as a membrane. It would be of interest to use the vitelline membrane technique (Wolff, 1960) with this organ to learn whether it could be well maintained if the membrane were provided for it.

The question of why mesonephros provides a more satisfactory situation for growth of the ovary than do the other organs tested cannot be answered on the basis of the present experiments. It might be explained either through the physical structure of the organ or its chemical activity. For human cancers, at least, the results from the separation of the cancer tissue from the mesonephros by a fold of vitelline membrane would seem to indicate that the effective factor is primarily chemical (Wolff and Wolff, 1961b).

The age of the organs or tissues used has a direct bearing on the results obtained. Explants from embryonic stages of amphibians readily grow and differentiate in culture. Larval organs grow, but continued differentiation of specialized cells, such as germ cells, is more difficult to obtain. Adult organ fragments are maintained with little change for some time, but ultimately there is cellular degeneration. Similar results have been reported by Steinberger et al. (1964) for growth of testicular tissue from rats of various ages.

Summary

Organs of the larval axolotl were grown either singly or in various combinations in watch glass cultures according to the method of Wolff and Haffen. They were maintained for 21 days. The organs cultured were ovary, testis, mesonephros, heart, liver, pancreas, spleen, brain, and pairs of ovary and testis with each of the others.

When organs are grown alone there is good maintenance of all organs except the liver. They might

TABLE 1.—Number of explants and time in culture.

Organ	Single cultures	Pairs-side by side	Pairs "sandwich"	Days in culture
Testis	4			4
"	18			10
"	5			21
"	30			21
Ovary	9			4-7
Heart	38			10
"	10			21
"	5			3-4
Liver	34			10
"	10			21
"	11			10
Mesonephros	10			21
"	9			21
Pancreas	5			21
Spleen	4			21
Brain		6		21
Testis & ovary		6		21
" " heart		6		21
" " liver		6		21
" " mesonephros		6		21
" " pancreas		5	10	21
Ovary & heart		11	7	21
" " liver		16	4	21
" " mesonephros		12	4	21
" " pancreas		11	5	21
" " spleen		5	5	21
" " brain				

be arranged in a series of decreasing competence in culture: testis, mesonephros, brain, spleen, pancreas, heart, ovary, liver. In some of these there is little difference between two mentioned in sequence as to the quality of their response to the culture situation.

Ovary-testis pairs in culture result in suppression of the germinal cells of the ovary.

Combinations of testis with liver, mesonephros or pancreas show little change in either organ of the pair from its condition when cultured alone. Some of the testes had lumina in a few seminiferous tubules when grown with mesonephros. In the testis-heart pairs neither organ appears to be quite as well maintained as each had been alone.

Combinations of ovary with mesonephros, brain and spleen are better maintained in pairs. There are few degenerating oocytes; young germ cells and non-germinal elements have proliferated. In combination with heart, the ovary does not show any improvement over culture alone. Ovarian tissue is destroyed when in combination with pancreas, in contrast to the condition of testis-pancreas pairs. Neither ovary nor liver are well maintained when these two organs are paired in culture.

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