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In vitro Studies on Differentiation of the Reproductive Tract

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In vitro studies on differentiation of the reproductive tract

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[Plate 16]

Contents

This paper will summarize some of the results of a series of organ-culture studies on sex differentiation of the reproductive tract of foetal guinea-pigs. Three aspects will be discussed: (1) the the secretory activity of the gonads in relation to the chronological steps in morphogenesis of the gonads and the reproductive ducts (Wolffian and Müllerian); (2) the dependence of the Wolffian ducts on testicular hormones at the critical period of development; (3) the marked independence of the Müllerian ducts from ovarian-secreted hormones.

A culture method was selected for this study because organs or reproductive tracts can be isolated from the complex foetal environment as explants and maintained in culture under controlled conditions, thus avoiding many complications encountered in experimentation *in vivo*. The explantation and culture of isolated reproductive tracts of foetal rats and mice have been successful (see review by Price & Ortiz 1965) and essentially the same method that was used in these earlier studies has been employed for the guinea-pig, except for some important modifications. The technique is basically a watch-glass method in which a natural medium is used. A detailed description was given in Zaaijer, Price & Ortiz (1966) and Price, Ortiz & Zaaijer (1967).

The guinea-pig is a favourable species for such experimentation because it is a long-term rodent (average gestation period 68 days) and the morphogenetic changes involved in sex differentiation of the reproductive ducts *in vivo* extend from 29 to 38 days of foetal life. The stages and timing in sex differentiation *in vitro* were found to be closely similar to those *in vivo*. This protracted period of 9 days was advantageous for close study *in vitro* of the steps in maintenance and development of the ducts or, conversely, in their retrogression and degeneration under various hormonal conditions. Such a study was greatly facilitated by the visibility of the ducts in the explants during the culture period.

Results

Secretory activity of the gonads

A bioindicator test was developed to detect the presence of hormones with androgenic activity in foetal gonads and other organs. The test consisted of co-culturing the foetal organ to be examined with a piece of the ventral prostrate gland of a young rat 21 to 28 days of postnatal age (see Zaaijer *et al.* 1966). The histological structure of the androgen-dependent prostate after 5 or 6 days of culture showed clearly whether or not an androgenic source was present. Retrogression always occurred in prostate explants cultured alone, which attested to the non-androgenicity of the medium.

Table 1. Results of androgenic tests on organs of foetal guinea-pigs cultured in contact with rat ventral prostate†

organs cultured with prostate	age at explantation in days p.c.	number of explants and response of prostate tissue		
		stimulated	incompletely retrogressed	retrogressed
testis	22–23	9	0	0
	25–36	53	0	0
	41–46	8	0	0
	60–62	14	0	0
ovary	22–23	0	0	6
	25–36	0	0	56
	41–46	6	8	1
	60–62	22	0	0
adrenal cortex	22–23	10	0	0
	25–36	103	0	0
	41–46	6	0	0
	60–62	28	0	0
mesonephros	22–23	0	0	10
metanephros	22–23	0	0	5
	25–30	0	0	19
urinary bladder	25–30	0	0	7
	30–36	0	0	5
anterior pituitary	60–62	0	0	16
rat prostate cultured alone	age at explantation (postnatal)			
	21–28	0	0	125

† Culture period 5 or 6 days. Data compiled from Zaaijer *et al.* 1966; Ortiz *et al.* 1966, 1967.

The results of culturing a series of foetal organs at ages of 22 to 62 days are summarized in table 1. The testes were secreting androgenic hormones at all ages that were tested. Notably, the gonad primordium at 22 to 23 days, undifferentiated as observed in routine histological preparations, proved highly androgenic when it differentiated in culture as a testis (figure 3, plate 16). The gonads, pieces of mesonephros, and adrenal cortical primordia were cut from the urogenital ridge at this stage, as shown in figure 2, plate 16, and cultured separately. This rather difficult dissection was necessary because it had been found in early experiments that when the whole ridge was explanted (figure 1, plate 16), the prostate was always strongly stimulated regardless

of the sex of the gonad. The androgenic source was identified as the small primordium of the adrenal cortex, which at this and all subsequent ages, was always highly androgenic. It should be noted that explants of the mesonephros, in common with several non-endocrine organs and the anterior pituitary, had no stimulating influence on the prostate (table 1).

The evidence for early secretion of foetal testicular hormones is in sharp contrast to the evidence for late inception of metabolic activity in the ovary as judged by the presence of androgenic hormones. No secretory activity of the ovary could be detected until 41 to 46 days, when some explants were positive, but at 62 days, all that were tested were androgenic. It was proposed (Ortiz, Zaaijer & Price 1967) that the ovarian secretions that had androgenic activity on prostate explants were probably androgenic hormones in the biosynthetic pathways leading to oestrogen secretion.

In terms of the chronological steps in organogenesis of the reproductive tract, androgenic hormones are secreted by the testis extremely early and are already present, for example, when Müllerian ducts are first beginning to form at 22 to 23 days. Thus, testicular androgen secretion considerably precedes the critical stage in Wolffian duct maintenance at 26 to 27 days, as discussed in the next section, and long precedes the beginning of degeneration of the Müllerian ducts at 29 to 30 days. In contrast, metabolic activity of the ovary as evidenced by secretion of hormones with androgenic action is delayed until after sex differentiation of the ducts has taken place and the Müllerian ducts have undergone marked morphogenetic and histogenetic differentiation.

The findings in the bioindicator tests on gonadal secretion and study of the steps in development *in vivo* indicated that two stages were very important for experiments in explantation of reproductive tracts. Accordingly, male and female tracts were explanted at the ambisexual period of 26 to 27 days when both sexes have a double set of reproductive ducts, and at the beginning of sex differentiation at 29 to 30 days when Müllerian ducts begin to retrogress in males, and Wolffian ducts, in females. The explants were observed and photographed during the culture period of 9 to 11 days and then fixed for histological study and comparison with normal chronological stages *in vivo*.

Dependence of the Wolffian ducts on testicular hormones

In brief, in male tracts explanted at the ambisexual stage (figure 4, plate 16) with a foetal testis present, the Wolffian ducts were retained (figure 6, plate 16); in those with the testis replaced by adrenal or by ovaries from a 60-day foetus, the Wolffian ducts were also retained. In explants lacking testes or a substitute androgen source, the Wolffian ducts retrogressed completely (figure 5, plate 16) and, significantly, they did so in the same pattern and at the same rate as the Wolffian ducts of females *in vivo*. In explanted female tracts, foetal testes, adrenals or pieces of ovary from a foetus 60 days old were also effective in maintaining the Wolffian ducts, which otherwise retrogressed as they do *in vivo*.

In male tracts explanted at 29 to 30 days, Wolffian ducts were retained when testes or other androgenic organs were present. However, in contrast to the findings at the younger stage, complete duct retrogression took place in some but not all explants in the absence of an androgenic organ. It was clear that in some tracts, stabilization of the ducts had been reached *in vivo* before explantation. In almost all female tracts that were explanted at this stage or slightly earlier, the Wolffian ducts underwent complete, or nearly complete, retrogression even when testes or adrenals were provided in culture. However, in three explants with testis or adrenal pieces against the tract (figure 11, plate 16) the ducts were maintained. These explants were

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still capable of responding to androgenic stimulation, but in the other tracts, retrogressive changes had already progressed too far *in vivo*.

These findings at the two stages in males and females serve to delimit the critical period of androgen-dependency of the Wolffian ducts in males and demonstrate that Wolffian ducts of females become androgen-dependent at precisely the same stage as those of males and are equally responsive to androgenic stimulation (Price *et al.* 1967; Ortiz *et al.* 1967).

Independence of the Müllerian ducts from ovarian-secreted hormones

The Müllerian ducts were retained in female tracts explanted at 26 to 27 days and at 29 to 30 days in the presence (figure 10, plate 16) or absence (figure 9, plate 16) of young ovaries and in the presence of androgenic organs—foetal testes, adrenals (figure 11, plate 16) or ovary at 60 days of age. The ducts were neither inhibited nor stimulated by endogenous androgenic hormones in culture, even at the ambisexual stage. The morphogenesis and histogenesis of the Müllerian ducts in explanted tracts under all hormonal conditions was most marked when culture was begun at 29 to 30 days. The simple Müllerian duct at explantation (figure 8, plate 16)

Description of plate 16

Figures 1 to 11. Development of the foetal guinea-pig reproductive tract in culture. Figures 1, 2, 3 and 7 illustrate the androgenic test. All other figures are of uncultured or cultured tracts. Figures 1, 4 to 6 and 8 to 11 are photographed on the culture medium, while figures 2, 3 and 7 are photomicrographs of histological sections. White arrow points to Wolffian duct; black arrow, to Müllerian duct. The figures are reproduced from the following sources with permission: 1 and 2 are reprinted from figures 9 and 8, respectively, in Ortiz *et al.* (1966); 3, 4, 5, 7, 9 and 10, from figures 4, 9, 10, 5, 7 and 8, respectively, in Price *et al.* (1969); 11, from figure 8 in Price *et al.* in *Hormones in development* (eds. M. Hamburgh & E. J. W. Barrington), New York: Appleton–Century–Crofts, in press.

Figure 1. Whole urogenital ridge explanted at 22 to 23 days and cultured for 5 days with a piece of rat ventral prostate (on left). The Wolffian duct and mesonephric tubules are conspicuous. Note the distended prostatic acini indicating androgenic stimulation. (Magn. × 25.)

Figure 2. Cross-section of the urogenital ridge *in situ* at 22 days to show the gonad (G), mesonephros (M) and adrenal cortical primordium (A) as they were separated for androgenic tests. (Magn. × 50.)

Figure 3. Gonad-prostate explant cultured for 5 days. The gonad, explanted at 22 days, differentiated as a testis (T) and the prostate (P) was stimulated, as shown by the high epithelium. (Magn. × 350.)

Figure 4. Left side of an uncultured reproductive tract from a male foetus at 27 days—the ambisexual stage of the reproductive ducts. (Magn. × 10.)

Figure 5. Male tract explanted from foetus 27 days old and cultured without testes for 11 days. Wolffian ducts are retrogressed. Note caudal remnants of Müllerian ducts, which are also present *in vivo* at 38 days of age. (Magn. × 10.)

Figure 6. Right side of a male tract explanted from foetus 27 days old and cultured with testes for 11 days Wolffian duct is maintained completely; caudal remnant of Müllerian duct is not visible. (Magn. × 10.)

Figure 7. Gonad-prostate explant after 5 days of culture. The gonad, explanted at 22 days, differentiated as an ovary (O) and the prostate (P) retrogressed. Note the low epithelium. (Magn. × 350.)

Figure 8. Left side of an uncultured reproductive tract from a female foetus 29 days old. (Magn. × 10.)

Figure 9. Right side of female reproductive tract explanted from a foetus 30 days old and cultured without ovary for 9 days. Note conspicuous growth and coiling of the Müllerian duct and retrogression of the Wolffian duct. (Magn. × 10.)

Figure 10. Left side of same tract as in figure 9, cultured with the ovary for 9 days. Note the same degree of growth and coiling of the Müllerian duct and retrogression of the Wolffian duct, as in figure 9. (Magn. × 10.)

Figure 11. Left side of female reproductive tract explanted from a foetus 29 to 30 days old and cultured for 9 days. In this case, the ovaries were removed at explantation and replaced by two pieces of adrenal cortex from the same foetus. Note extensive growth and coiling of the Müllerian duct and complete maintenance of the Wolffian duct. The contralateral ducts were equally well developed. (Magn. × 25.)

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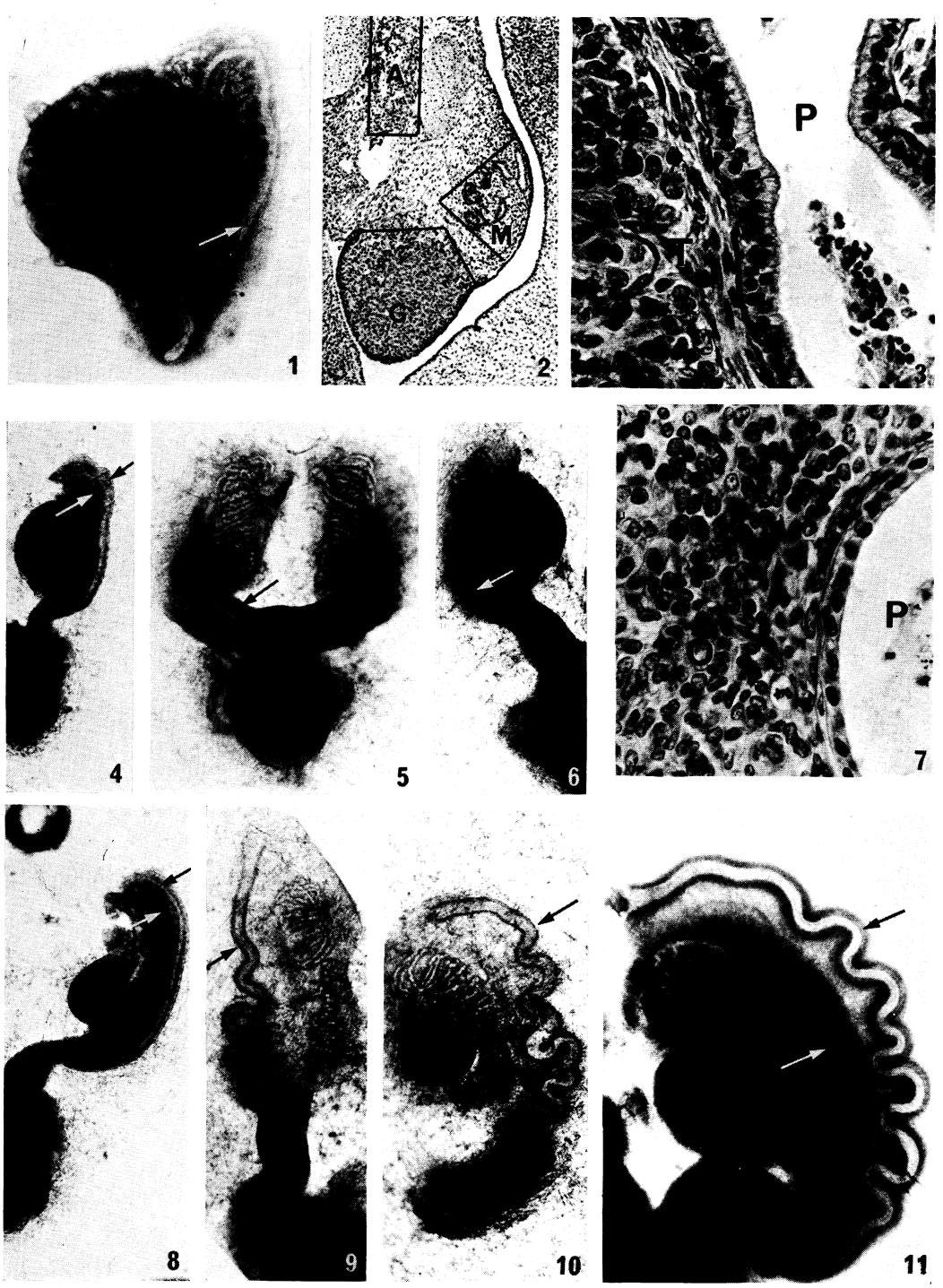

FIGURES 1–11. For description see facing page.

(*Facing p.* 136)

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underwent during culture the typical coiling of the oviductal region (figures 9 to 11, plate 16) and developed fimbriae around the ostium. These and other morphogenetic and histogenetic changes progressed at the same pace (although not to the same degree) as in oviducts *in vivo* (Price, Zaaijer & Ortiz 1968). The amount of growth and differentiation of the oviductal segment of the Müllerian duct in explanted reproductive tracts was all the more impressive because it occurred in the presence or absence of the young ovary and in the presence of androgenic organs which had stimulating effects on Wolffian ducts in the same explant (figure 11, plate 16).

It should be recorded here that Müllerian ducts in explants of male tracts retrogressed under all conditions even when explanted at the ambisexual stage. If they normally retrogress in the male because of the inhibitory influence of testicular hormones, they must already have been conditioned to retrogress before the ambisexual stage. This is a possibility.

Discussion

The results of these experiments have advanced our understanding of sex differentiation in the foetal guinea-pig by: (1) establishing that testes secrete androgenic hormones extremely precociously; (2) pinpointing the stage at which Wolffian ducts become androgen-dependent, a stage that marks their change-over from nephric ducts to excurrent ducts of the male reproductive system; (3) delimiting the critical stage for Wolffian duct maintenance; and (4) demonstrating the striking degree of hormone independence of the female Müllerian ducts.

Discussion of all these points in relation to other studies *in vitro* and *in vivo* is beyond the scope of this paper. Various aspects have been treated in our previous publications. A recent paper (Price, Zaaijer & Ortiz 1969) dealt with the role of foetal testicular hormones in modifying the expression of genetic factors in sex differentiation of the reproductive ducts. Only the precocious secretory activity of the testes will be discussed here.

The observation of secretion of testicular androgens as early as 22 to 23 days of age brings up the questions of the characterization of these androgenic hormones and whether they play any part in the early differentiation of the testis itself. Histochemical studies of Price, Ortiz & Deane (reviewed in Price & Ortiz 1965) showed that 3β-hydroxysteroid dehydrogenase activity was present in the interstitial cells of testes 28 to 50 days of age but no younger stages were examined. No enzyme activity was detected in ovaries during the same period of development. These findings established the capability of the testis to secrete steroids at the time when sex differentiation is occurring in the reproductive ducts and in accessory glands and suggested that the androgens that we detected might be steroidal. Bloch (1967*a*, *b*) then showed that homogenates of guinea-pig testes at mid- and late gestation converted 7-[^{3}H]pregnenolone and 4-[^{14}C]progesterone to testosterone and androstenedione. With this, the case was strengthened for the probable identity of the normally secreted testicular androgens as C_{19} steroids, at least at the stage of rapid sex differentiation of ducts and glands.

In recent experiments, R. W. Verhoef-Bouwknegt & J. J. P. Zaaijer (unpublished observations) found the localization of 3β-hydroxysteroid dehydrogenase in gonads 23 to 24$\frac{1}{2}$ days old. The crucial point is that enzyme activity was present in those cases in which culture of the contralateral gonad with prostate showed that the gonad was a testis and, furthermore, that it was secreting androgenic hormones. This sets back the time of capability of secretion of steroids in the testis to the stage when androgens are being secreted in testes that appear either completely

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undifferentiated in routine histological preparations or are just beginning to develop faint medullary cords.

Strong evidence of another sort for the ability of 'undifferentiated' testes of foetal guinea-pigs to secrete steroidal hormones has been provided by Black & Christensen (1969) from electron-microscopical studies. They found areas of smooth endoplasmic reticulum in interstitial cells and presumptive Sertoli cells of testes 22 to 24 days of age. Similar agranular endoplasmic reticulum had been described in the interstitial cells of adult guinea-pig testes by Christensen (1965) and he had proposed from his studies and the biochemical findings of others that this reticulum is the site of at least two enzymes that are important in androgen biosynthesis. Black & Christensen found only patches of smooth endoplasmic reticulum in the interstitial cells at 22 to 24 days and concluded that the cells were not completely differentiated but that they evidently possessed the capability for biosynthesis of androgens before they were completely differentiated morphologically. They added the important observation that when thin sections of plastic-embedded gonads were studied by light microscopy, primitive sex cords could be seen in very early stages of development at 21 to 22 days.

The findings of Black & Christensen and our results on androgenic tests demonstrate that at a very early stage the testes of foetal guinea-pigs are equipped to secrete androgenic steroids when they are, indeed, producing androgens. It should be pointed out that the only sex differentiation that is occurring at this time is in the gonads themselves. In the guinea-pig, many primordial germ cells have migrated into the germinal epithelium of the urogenital ridge by 19 days and it now appears that in all probability the somatic tissues of prospective testis and ovary differ functionally very soon after this time.

The observation of secretory activity in testes that are in the first stages of sex differentiation coupled with the findings of the beginning of special morphogenesis in interstitial cells (and presumptive Sertoli cells) does not provide evidence that the testis is secreting androgenic steroids. Nor does it suggest that our uncharacterized androgenic hormones, or androgenic steroids, or any other steroids, are necessarily promoting testicular differentiation. However, it does point out the fact that foetal testes are capable of secreting androgens and synthesizing steroids at far younger stages than has commonly been recognized. It does serve to re-emphasize the enormous importance of the somatic tissues of the 'undifferentiated' gonad. It seems clear that in individuals that are genotypically XY or XX the sex-determining factors that govern the differentiation of testis or ovary must operate in some way through the somatic tissues of the gonads, and it is somatic tissues which direct, by regional environmental cues, the differentiation of germ cells (which obviously have no special sex-determining factors) in the pathways to oogenesis or spermatogenesis. Our experiments have added in some measure to knowledge of the secretory ability of 'undifferentiated' testes but far more information is needed before the method by which sex-determining factors act in gonadogenesis can be understood or any estimate can be made of the numbers and types of testicular secretions.

These studies have been done in collaboration with J. J. P. Zaaijer, University of Leiden, and E. Ortiz, University of Puerto Rico. They have been aided by research grants HD-03471 and AM-03628 from the National Institutes of Health, United States Public Health Service, and by the Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek.

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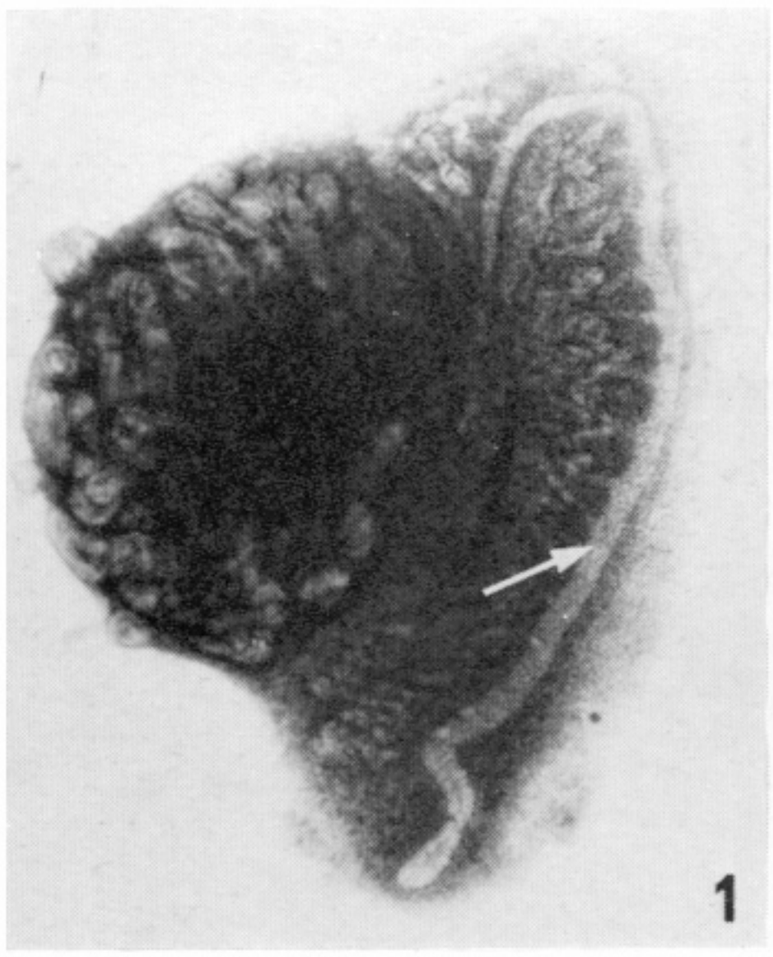

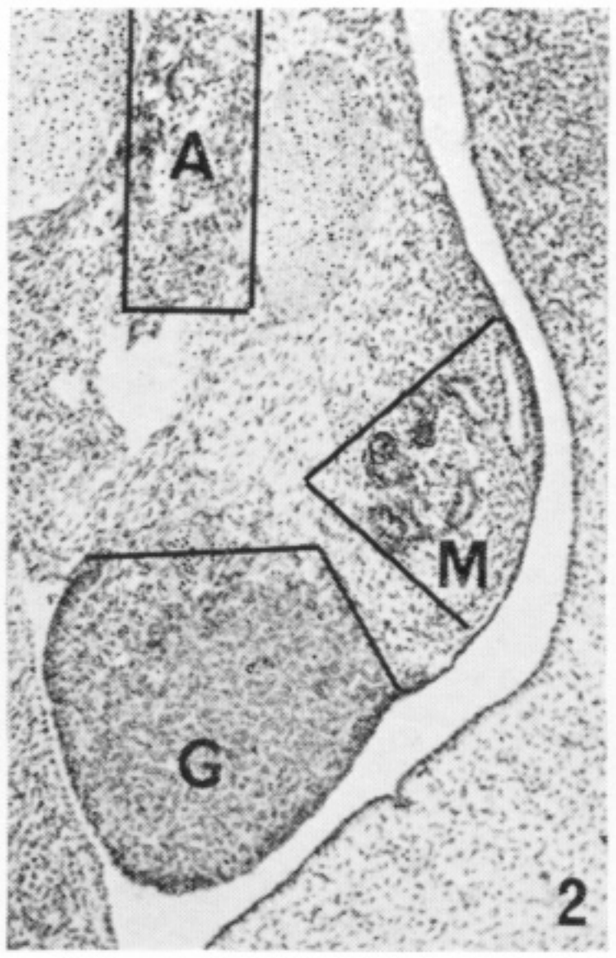

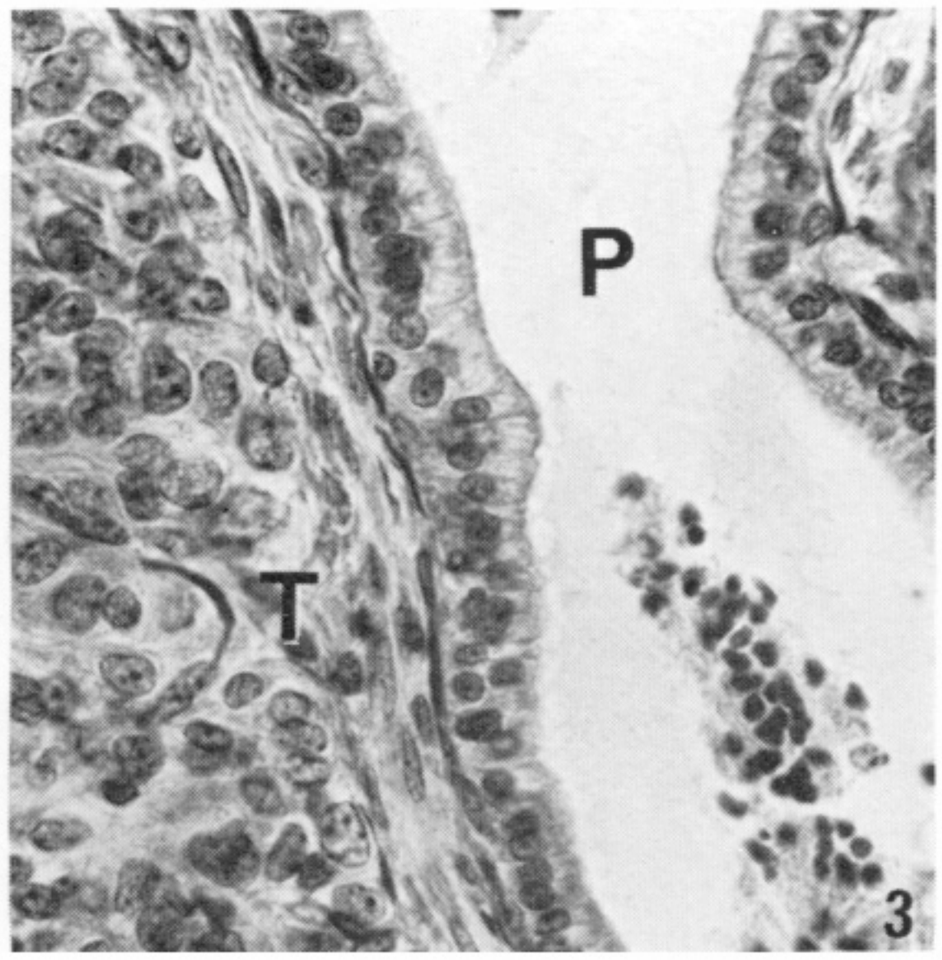

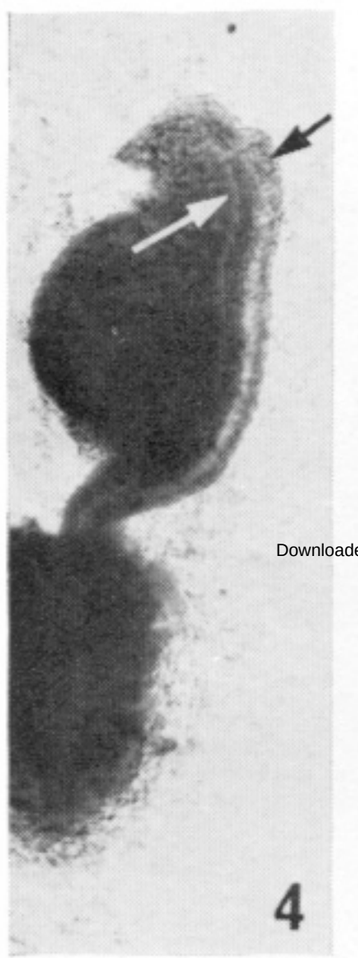

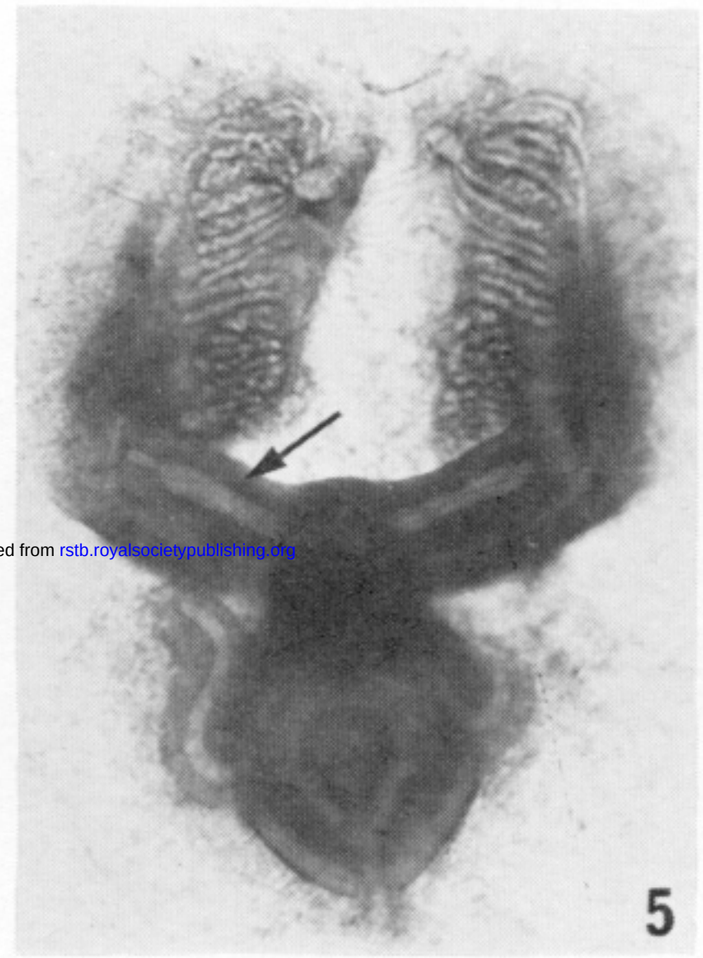

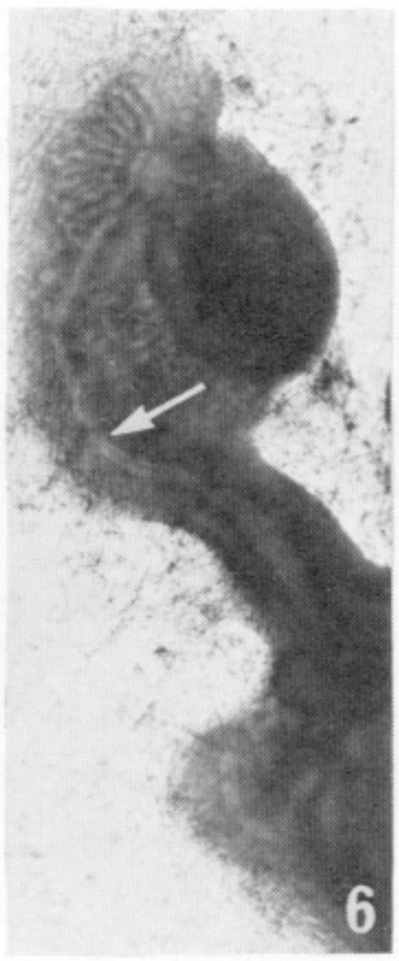

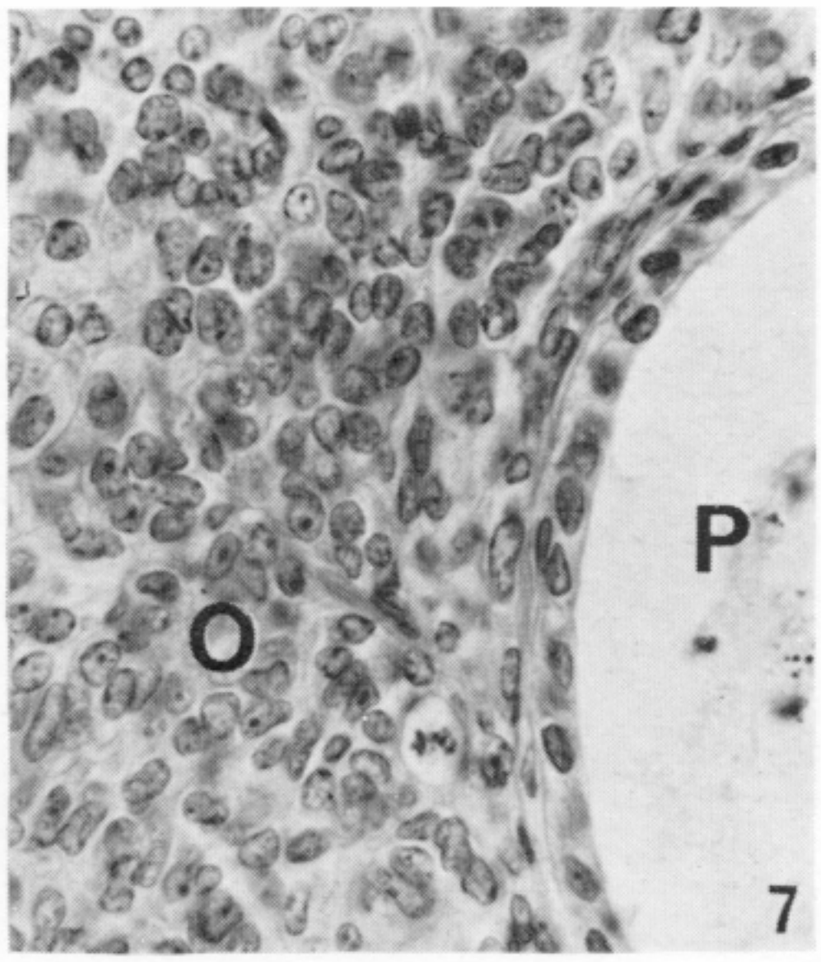

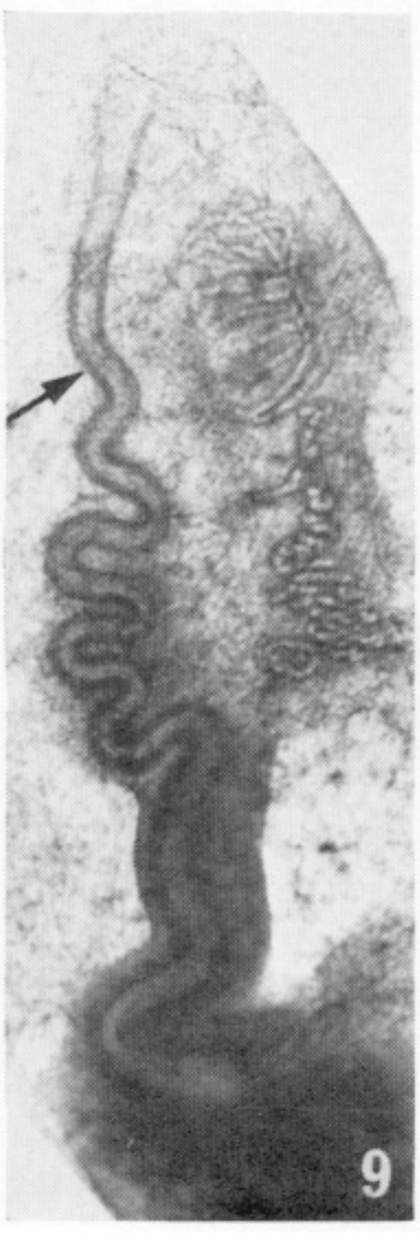

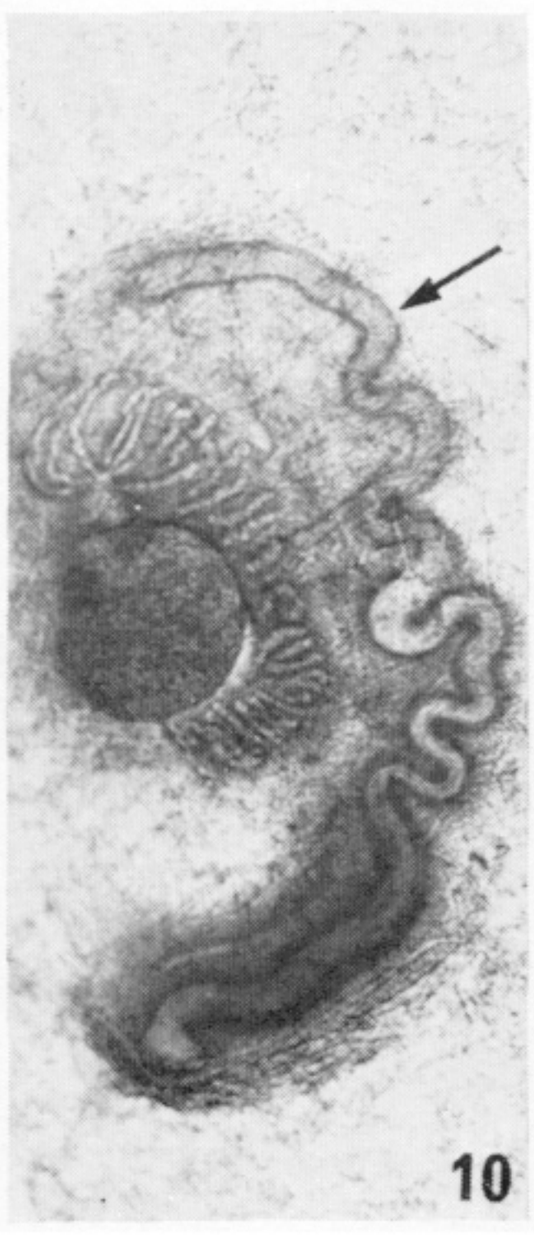

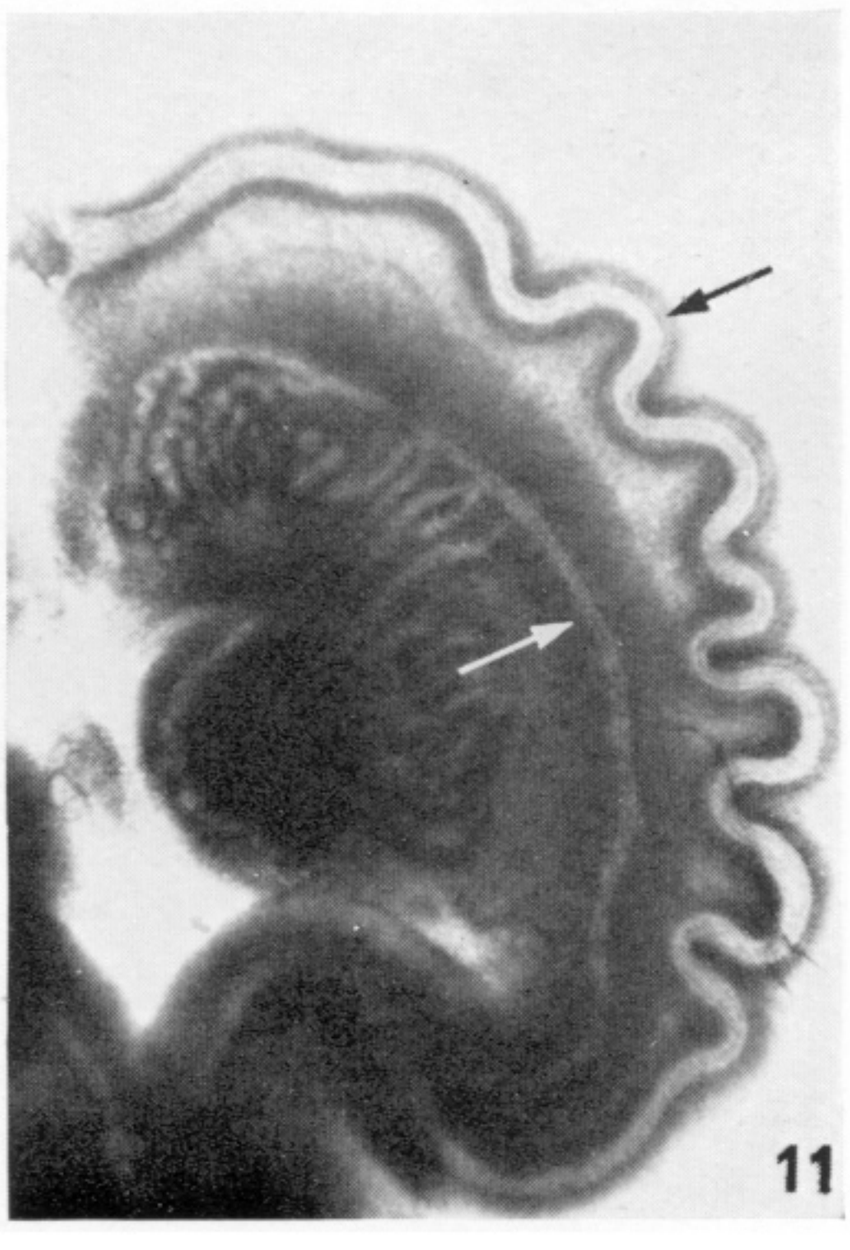

FIGURES 1–11. For description see facing page.