The role of the Wolffian ducts in the formation of the sinus vagina: an organ culture study

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SUMMARY

In mammals formation of a sinus vagina is inhibited in the male by endogenous testosterone from the embryonic testes. To answer the question which morphogenetic events during formation of the vagina are influenced by testosterone, we explanted genital tracts of mouse embryos in the indifferent stage of development in organ culture. Half of the explants were treated with testosterone and therefore developed in male direction. The other half was kept without testosterone and developed constitutively in female direction. Since the antiMüller factor was not present, in both types of cultures the Müllerian ducts were preserved.

During female development the Müllerian ducts fused with the dorsolaterally apposed caudal segments of the Wolffian ducts. Thus the caudal segments of the Wolffian ducts were incorporated in the vaginal plate, while cranially the Wolffian ducts degenerated as expected.

During male development fusion between Müllerian and Wolffian ducts did not occur. Under the influence of testosterone the respective caudal segments of the Wolffian ducts were surrounded by dense mesenchyme and further male differentiation took place.

We conclude that the 'sinus protrusions' or 'sinovaginal bulbs' observed during development of the vagina, are in fact the caudal segments of the Wolffian ducts. They serve as a link between Müllerian ducts and urogenital sinus. Formation of a sinus vagina is prevented by testosterone simply by induction of male development in this area.

INTRODUCTION

The development of the vagina in man and other mammals has been studied extensively and repeatedly since the beginning of descriptive embryology. The descriptions are governed by the controversy about the derivation of the vagina. The various theories comprised derivation from the Müllerian ducts, from the Wolffian ducts, from the urogenital sinus, or from combinations of these structures. Today, in general, it is held that the upper part of the vagina is a derivative of the fused Müllerian ducts (Müllerian vagina), whereas the lower part is a derivative of the urogenital sinus (sinus vagina) (Forsberg, 1963; Jost, 1971; O'Rahilly, 1977).

In our present organ culture study we observed that in the mouse embryo the lower segments of the Wolffian ducts were incorporated in the vaginal plate. As will be shown below, this observation also has a bearing on the interpretation of

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vagina development in the human. It will be discussed in the light of the formerly widely held view of a contribution of the Wolffian ducts to the vagina. We conclude from our observation that the 'sinovaginal bulbs' or 'sinus protrusions' described in man and other mammals, are in fact the caudal segments of the Wolffian ducts.

In general terms the vagina in mammals develops as follows: in the indifferent stage of the genital ducts, the fused Müllerian ducts contact the wall of the urogenital sinus by means of a solid epithelial tip which merges with the epithelium of the dorsal wall of the sinus and bulges into its lumen. The solid epithelial structure is known as the Müllerian tubercle. Laterally and slightly caudally from the Müllerian tubercle, the Wolffian ducts enter the urogenital sinus. In contrast to the Müllerian ducts, the Wolffian ducts open freely into the sinus. During female development the mouth of the Wolffian ducts is transformed into an epithelial pocket carrying the degenerating Wolffian ducts at its cranial end. The bilateral pockets proliferate and form two solid epithelial bulbs, the so-called sinovaginal bulbs, extending as dorsolateral projections from the urogenital sinus. Cranially the bulbs fuse with the already fused Müllerian ducts. The Müllerian tubercle disappears or becomes incorporated into the solid vaginal plate which forms by fusion of the two bulbs with each other. Later on in the solid vaginal plate a lumen forms by necrosis of the central cells. In the mouse the sinovaginal bulbs are represented by open duct-like extensions of the urogenital sinus and were called 'sinus protrusions' by Forsberg (1963).

The endocrine regulation of vaginal development is as follows: in the male the antiMüllerian factor produced in the testes induces regression of the Müllerian ducts and thus prevents formation of a Müllerian vagina (Josso, 1973). The development of a sinus vagina is inhibited by testosterone. The latter notion is based on the following observations: exogenous androgens administered during pregnancy, inhibit the formation of a sinus vagina (Raynaud, 1942, 1950). Treatment with antiandrogens leads to development of a sinus vagina in male embryos (Neumann et al. 1975). In the syndrome of testicular feminization (Tfm) in the mouse, characterized by general androgen insensitivity, the Müllerian vagina is inhibited whereas a sinus vagina is formed (Lyon & Hawkes, 1970).

In our study we were interested in the morphogenetic mechanism leading to the formation of a sinus vagina and in the relationship of this process to the abnormal course of the male genital ducts in intersexes. In intersexes produced by sex reversal of Tfm heterozygotes the Wolffian ducts and the excretory ducts of the vesicular glands run down behind the sinus vagina and open into the urethra together with the vagina at the level of the penile bulb (Drews, 1975).

The present organ culture experiment was designed to show the effect of testosterone on the formation of the sinus vagina. Embryonic mouse genital tracts were explanted in the indifferent stage of development. Half of the cultures were treated with testosterone and therefore developed in male direction.
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The other half were kept without testosterone and developed constitutively in female direction. Since the testes were not included in the explants, the antiMüller-factor was not present. Correspondingly, the Müllerian ducts were maintained and developed equally well in both types of cultures.

In explants kept without testosterone, the Müllerian ducts fused with the dorsolaterally apposed Wolffian ducts and thus allowed vaginal development to proceed. In explants kept with testosterone, no fusion occurred. Formation of a sinus vagina was prevented by preservation of the integrity and further differentiation of the Wolffian ducts.

In addition to genital tracts from normal mouse embryos, we also explanted genital tracts from female embryos heterozygous for testicular feminization (X_Tfm/X) in order to observe the expression of the inherent mosaic of androgen-sensitive X^+ cells and androgen-insensitive X_Tfm cells. In one explant the expression of the mosaic was observed: female development characterized by fusion of Wolffian and Müllerian ducts, alternated with male development represented by non-fusion and maintenance of the integrity of the Wolffian duct.

MATERIALS AND METHODS

Female NMRI mice obtained from a local commercial breeder or females from our own Tfm-mouse colony were mated to NMRI males. Embryos were recovered on days 15, 16 and 17, the day of plug detection being day 1.

The sex of the embryos was determined by inspection of the gonads. The genital tracts were excised in Tyrode solution. Specimens were cleared ventrally and laterally from adhering tissues to render them as transparent as possible. They were trimmed by cutting off the upper parts of the genital ducts some distance above the bladder. Then the stem of sinus was cut at the level of the symphysis. The bladder itself was removed. The genital tracts were explanted with the dorsal or the lateral side up. The live explants were photographed daily through a dissecting microscope with a stereoadapter.

The culture medium used was Dulbecco's MEM with 10 % horse serum, 10 % chick embryo extract and addition of Pen-Strep (50 units/ml). In part of the organ cultures, 10^{-6} \text{M}-testosterone was added to the medium (see Table 1). Instead of millipore filters used in earlier studies, a piece of a 1 % agar layer was used as support for the explants at the medium–air interface. The incubator was gased with 5 % CO_2.

At the end of the culture period of up to 8 days, explants were fixed in Bouin's solution, oriented with stained agar blocks according to Arnolds (1978), and embedded in Paraplast. Serial sections were prepared and stained with haematoxylin–eosin and HOPA (Haematoxylin, Orange G, Phosphomolybdenic acid, Aniline blue) after Tonutti (Tonutti et al. 1960).

The reconstructions were compiled from serial sections with the Perspectomat
Fig. 1. Stereophotographs of a living explant from day 15 of pregnancy kept without testosterone, on the first day (A), on the third day (B) and on the fifth day (C) in culture (Exp. No. 13, Table 1). Female development. The outlines of the genital ducts include Wolffian and Müllerian ducts. Fusion and secondary elongation of the Müllerian ducts is visible on day 3 in culture. Downgrowth of the vaginal anlage is evident, particularly when compared to male development shown in Fig. 2. The lower part of the urethra begins to contract rhythmically after a culture period corresponding to day 17 of pregnancy. The explant lies with the dorsal side up on the agar. ×44.
Fig. 2. Stereophotographs of a living explant from day 15 of pregnancy kept with testosterone on the first (A), on the third (B), and on the fifth day (C) in culture. Male development. The Müllerian ducts are preserved and fusion is visible as in female development. Elongation and downgrowth do not occur. Enlargement and thickening of the sinus ridges is followed by outgrowth of multiple prostatic buds and appearance of vesicular glands. The explant lies with the dorsal side up on the agar. \( \times 44 \).
The following description is based on the list of cultures in Table 1. As indicated, the genital tracts were explanted on days 15, 16 or 17 of pregnancy, the day of plug detection being day 1. The culture period is given in days corresponding to days of pregnancy. At the time of explantation, the testis and the ovary were clearly distinguishable by inspection in the dissecting microscope. Correspondingly, explants from embryos with ovaries were designated as XX and those from embryos with testes as XY. In each experimental series, half of the explants were cultured with testosterone and the other half without testosterone, so that treated and untreated explants from the same litter could be compared directly. Male embryos (XY) were always included in the group with testosterone. Comparable untreated and treated explants were fixed for histology in daily intervals covering the entire culture period.

For the sake of clarity we will separately describe the morphology of the explants in the indifferent stage at the beginning of the culture period, then the female development of explants cultured without testosterone, the male development induced by testosterone, and finally the observations in Tfm heterozygotes. The description refers to stereophotographs of the live explants taken in daily intervals, to the serial sections, and to the representative graphic reconstructions thereof.

**Indifferent stage**

In the mouse the Müllerian ducts reach the urogenital sinus at day 14 of pregnancy. In the respective area beneath the bladder, the sinus is typically U shaped in cross section with two sinus ridges or dorsal urethral folds facing the rectum (Forsberg, 1963). The Wolffian ducts enter the sinus ridges dorsally, whereas the Müllerian ducts contact the sinus ridges slightly cranially and medially from the latter. In this region it is not before day 16 that a sex difference becomes visible.

After excision of the genital tract from the embryo on day 15 or 16 of pregnancy, the area of contact between Wolffian and Müllerian ducts with the urogenital sinus appeared as a characteristic rhombic figure when viewed dorsally in dark-field transmission illumination through the dissecting microscope. The lower crura were formed by the sinus ridges, whereas the upper crura corresponded to the superposed epithelial outlines of both genital ducts. Several hours after explantation the explants flattened and the tissue again became translucent. Fig. 1A and 2B show explants of day 15 photographed at the day of
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expansion. Both were explanted with the dorsal side up. The rhombus formed by the sinus ridges and the genital ducts can be recognized. Individual Wolffian and Müllerian ducts however cannot be designated with certainty. Both explants represent the indifferent stage from which female development starts off in cultures without testosterone (Fig. 1) and male development in cultures with testosterone (Fig. 2).

To visualize the exact position of the ducts at the beginning of the culture period, three explants of day 15 and two of day 16 were fixed and processed for histology on the same day after they had been oriented on the agar and grown for about 5 h (Table 1). Figure 3 shows a specimen of day 16 explanted with the dorsal side up. In the centre, two closely apposed Müllerian ducts and two dorsolaterally located Wolffian ducts can be seen just before entering the sinus. Probably due to damage during cutting and handling, some cells in the mesenchyme undergo necrosis whereas in the epithelium of the ducts mitotic figures are visible.

Figure 7 shows a graphic reconstruction of a specimen explanted at day 16 of pregnancy and fixed for histology the same day. The ducts are still in the indifferent stage of development. The specimen is comparable to those of day 15. The reconstruction shows only the upper half of the rhombus visible in the living explants (compare Fig. 1A and 2A). The upper crura of the rhombus are formed by the superposed Wolffian of Müllerian ducts.

Development without testosterone

During female development in vivo, the Müllerian ducts fuse in the midline and the Wolffian ducts degenerate. Degeneration of the Wolffian ducts starts on day 16 of pregnancy midway between the gonads and the point of contact with the sinus and proceeds cranially and caudally up to day 19.

Our cultures kept without testosterone developed constitutively in the female direction. Although only the lower ends of the genital ducts were included in the explants, fusion of the Müllerian ducts and degeneration of the Wolffian ducts followed the same time schedule as in vivo. In the living explants, fusion and enlargement of the Müllerian ducts was clearly visible after 2 days in culture (Fig. 1B). The fused lower segments of the Müllerian ducts appeared as a bell-shaped central thickening in the upper half of the rhombus visible in the preceding indifferent stage. With further development two crura emerged from the lower base of the bell-shaped structure connecting the latter with the lateral sinus ridges (Fig. 1B and C). The crura represented the Müllerian ducts elongating caudally to fuse with the lower segments of the Wolffian ducts. A clear outline of the Wolffian ducts was not visible in the living explants; they were hidden in the pronounced bell-shaped structure of the fused Müllerian ducts. From the histology of the explants described below, it became clear that of the caudal straight portions of the Wolffian ducts included in the explants, the lower two thirds remained intact and finally fused with the Müllerian ducts.
Table 1. *List of explants and time schedule of cultivation*

The explants described belong to four experiments conducted on days 15, 16 and 17 of pregnancy. Explants from embryos with ovaries are designated as XX and those from embryos with testes as XY or *Tfm/Y* when carrying testicular feminization. They are listed on the day of interruption and fixation for histology. The time schedule shows that each day is represented by comparables cultures kept without testosterone (0T) and with testosterone (+T).

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Culture period corresponding to days of pregnancy

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Wolffian ducts and formation of sinus vagina

The lower half of the rhombus seen in the living explants and representing the sinus ridges, was transformed into a cup-like structure into which the fused Müllerian ducts sank down (Fig. 1B and C). When oriented in a lateral position on the agar (not shown) the cup of the sinus ridges carrying the genital ducts bulged dorsally like a backpack at the neck of the urethra. This characteristic configuration appears also in vivo during female development.

In the living explants, development could be followed easily up to day 19 (Fig. 1C). On prolonged culture periods as in Exp. No. 13, Table 1, the explants became opaque and clumsy in outline although the histological structure was still well preserved. On day 17 in most of the explants the caudal part of the urethra started to contract rhythmically. The contractions finally pulled the urethra into an abnormal ventrally bent position (Fig. 1C and 2C). The contractions were independent of testosterone and have already been described by Cunha (1973). They are, however, not generated by a smooth muscle layer as stated by Cunha, but by the cross-striated urethral muscle of the mouse (Thiedemann & Drews, 1980).

The characteristic features of the explants grown without testosterone were secondary elongation of the fused Müllerian ducts, preservation of the caudal segments of the Wolffian ducts, and finally longitudinal fusion of Wolffian and Müllerian ducts. The fusion of Wolffian and Müllerian ducts is demonstrated in the serial sections of a day-19 explant after 3 days in culture (Fig. 5). Cranially the degenerating remnants of the Wolffian ducts are found in a dorsolateral position attached to the enlarged and fused Müllerian ducts (Fig. 5A). More caudally, the Müllerian ducts segregate again (Fig. 5B). The Wolffian ducts gradually enlarge and the cuboidal epithelium with signs of degeneration is replaced by high columnar epithelium showing many mitoses. Concomitantly, the Wolffian epithelium acquires the characteristic appearance of Müllerian

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Fig. 3. Histology of an explant from day 16 of pregnancy after 5 h in culture. Indifferent stage of development. The closely apposed genital ducts and a fragment of the urogenital sinus are visible. Wd = Wolffian duct; Md = Müllerian duct; ugs = urogenital sinus. ×95.
Fig. 4. Histology of an explant from day 16 of pregnancy after 1 day in culture with testosterone. (A) Level of fused Müllerian ducts. (B) On the left side of the explant Wolffian and Müllerian ducts fuse with each other thus indicating the presence of a X<sup>Tfm</sup>/X<sup>+</sup> mosaic. (C) Entrance of the ducts into the urogenital sinus. The explant was oriented in a lateral position. The corresponding graphic reconstruction is shown in Fig. 8. ×95. fMd = fused Müllerian ducts; Md = Müllerian duct; Wd = Wolffian duct; ugs = urogenital sinus.
epithelium and after fusion with the latter, the contribution of Wolffian epithelium to the vagina can be hardly identified except by location (Fig. 5B and C). The fused Wolffian and Müllerian ducts merge with the solid sinus ridges. Thereby the original free opening of both Wolffian and Müllerian ducts into the urogenital sinus is lost (Fig. 5D).

Female development of the explants is visualized by the reconstructions in Figs 7–10, which were all prepared in the same scale from serial sections of specimens of experiment No. 6 (Table 1). Figure 7 shows a specimen in the indifferent stage of day 16 fixed on the day of explantation. The specimen of Fig. 8 was treated with testosterone and expressed the phenotype of a Tfm heterozygote. In this context it may be used to demonstrate fusion and enlargement of the Müllerian ducts at day 17 which occurred likewise in treated and untreated cultures. Figure 9 shows an explant of day 18. In this case major asymmetries developed probably due to inadequate orientation on the agar. At the left side the contact between the lower end of the Wolffian duct and the left Müllerian duct is not established, whereas on the right side fusion occurred regularly. The cranial portions of the Wolffian ducts have already regressed. Figure 10 is the reconstruction of the female specimen of day 19, the serial sections of which are shown in Fig. 5A–D. In the Wolffian ducts two parts can be distinguished. In the degenerating upper parts the ducts are small and taper off cranially. The lower parts are enlarged and fuse over their whole length with the elongating roots of the Müllerian ducts. The fused ducts of both sides merge with the dorsal sinus ridges, which have developed into prominent 'buttocks' at the dorsal aspect of the urethra (compare the living explants in Fig. 1C).

**Development with testosterone**

The development of the explants treated with testosterone corresponded to male development in vivo with one important exception: the anti Müllerian factor was missing and therefore the Müllerian ducts were maintained and developed as in the female. This fact was constitutive for our experimental design. It allowed close comparison of the male and the female phenotype by using the Müllerian ducts as a landmark common to both types of explants. On the other hand, differences observed in the development of the sinus vagina were restricted to effects of testosterone.

In general, cultures treated with testosterone exhibited a better overall growth (compare Fig. 1 and 2). Mesenchymal and epithelial tissues appeared more healthy. Necrotic areas in the centre of the cultures were less frequently observed and were less extended. Even the fused Müllerian structures seemed to profit from trophic influences exerted by testosterone.

After 2 days in culture, corresponding to day 17 of pregnancy (Fig. 2B), the central bell-shaped outline of the fused Müllerian ducts was clearly visible as in female development. The sinus ridges also transformed into a cup-like structure. The projection of the combined sinus ridges dorsally and laterally can easily be
seen in the stereophotographs of Fig. 2B and C. However, formation of segregating crura and caudal elongation of the Müllerian ducts did not occur. In contrast to the development without testosterone, the fused Müllerian ducts did not move down between the sinus ridges. Instead, at the upper rim of the cup formed by the ridges, a number of epithelial buds appeared which were well developed after 4 days in culture (Fig. 2C). The buds represented the vesicular glands arising from the base of the Wolffian ducts and the prostate glands formed by the adjacent sinus epithelium.

The histology of the explants cultured with testosterone is shown in Fig. 6A–D. The respective explant stems from experiment No. 6, day 19, and is directly comparable with the explant of Fig. 5A–D which stems from the same litter and the same day the only difference being treatment with testosterone. During male development the Wolffian ducts were maintained throughout the whole length of the specimens in about the same size as the Müllerian ducts. In contrast to female development no fusion occurred between Wolffian and Müllerian ducts. After day 17, the Wolffian epithelium showed signs of stimulated growth with enlargement of the ducts and appearance of many mitotic figures. On day 18 the buds of the vesicular glands appeared as thickening and doubling of the epithelial lining (Fig. 6B, C). On day 19, the vesicular glands formed flat cap-like protrusions at the dorsomedial aspects of the Wolffian ducts. In Fig. 6B and C and the corresponding reconstruction Fig. 11 the buds are still included in the Wolffian epithelium and extend over the whole length of the lower segments of the ducts. The explant of experiment 13 (Table 1) shown in Fig. 13 and in the stereophotographs of Fig. 2C is slightly further developed. The tips of the vesicular glands have now separated from the Wolffian ducts.

Without testosterone the Müllerian ducts elongated caudally by fusion with the dorsolaterally located Wolffian ducts. With testosterone this fusion did not occur. By the enlarging Wolffian structures the Müllerian ducts were pushed together and restricted to the original point of contact with the sinus. In cross sections they formed a characteristic triangular roof over the dorsal aspect of the urethra (Fig. 6A, B), wedged in between the expanding Wolffian ducts and the vesicular glands. Besides the changes in the epithelial ducts we observed also

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Fig. 5. Histology of an explant of day 16 of pregnancy grown for 3 days in culture without testosterone. Female development. (A) Fused Müllerian ducts with dorsolaterally attached degenerating Wolffian ducts (on the right the specimen was damaged after fixation). (B) The Müllerian ducts segregate again. The caudal portions of the Wolffian ducts are well preserved. (C) Wolffian and Müllerian ducts fuse. (D) The combined ducts merge with the sinus ridges. The graphic reconstruction of the explant is shown in Fig. 10. Abbreviations as in Fig. 4. ×95.

Fig. 6. Serial sections of an explant of day 16 of pregnancy grown for 3 days in culture with testosterone. Male development. (A) Wolffian and fused Müllerian ducts show stimulated growth. (B) and (C) On the dorsal aspect of the Wolffian ducts the buds of the vesicular glands appear. The Müllerian ducts form a triangular roof at their original point of contact with the urogenital sinus. (D) From the sinus ridges (sr) prostate buds arise. The graphic reconstruction of the explant is shown in Fig. 11. ×95.
Fig. 7. Graphic reconstruction of an explant of day 16 in the indifferent stage fixed at the day of explantation. The reconstructed epithelial structures correspond to the upper half of the rhombus seen in the living explants of Figs 1A and 2A. The reconstructions Figs 7–11 are taken from experiment No. 6 (Table 1) and drawn to the same scale. Md = Müllerian duct; Wd = Wolffian duct; ugs = urogenital sinus; sr = sinus ridges.

Fig. 8. Graphic reconstruction of an explant from day 16 of pregnancy cultured for one day with testosterone. Intersex development. The area of fusion between the left Wolffian and Müllerian duct indicates the presence of androgen insensitive X\textsuperscript{fm}\textsuperscript{+} cells in this region. Same explant as in Fig. 4.

Fig. 9. Graphic reconstruction of an explant from day 16 of pregnancy kept for 2 days in culture without testosterone. Female development. Due to inadequate orientation on the agar the contact between the left Wolffian and Müllerian duct is not established, whereas on the right side fusion of the ducts occurred regularly.
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Fig. 10. Graphic reconstruction of an explant from day 16 of pregnancy kept for 3 days in culture without testosterone. Female development. The fused Müllerian ducts segregate again and elongate caudally. The lower ends of the Wolffian ducts fuse longitudinally with the Müllerian ducts, while the upper parts degenerate. Same explant as in Fig. 5.

Fig. 11. Graphic reconstruction of an explant from day 16 of pregnancy kept for 3 days in culture with testosterone. Male development. Fusion between Wolffian and Müllerian ducts does not occur. The Wolffian ducts are well preserved and the appearance of vesicular glands is indicated by a dorsal thickening of the epithelium. Same explant as in Fig. 6.
differences in the behaviour of the mesenchyme. In explants kept with testosterone densely packed circular mesenchyme appeared around the Wolffian ducts which was not present in explants cultured without testosterone (compare Fig. 5C and 6C).

In the reconstructions male development can be followed starting out from the indifferent stage of Fig. 7 through the stage with fused Müllerian ducts as in Fig. 8, to the fully expressed male phenotype in Fig. 11. Although Fig. 8 represents a Tfαm heterozygote as described below, it can be used to visualize the general development of Wolffian and Müllerian ducts on day 17. Comparison of Fig. 11
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with the corresponding female stage in Fig. 10 demonstrates the enlargement of the Wolffian ducts, the lack of fusion with the Müllerian ducts, and the compression and retention of the Müllerian ducts at their original point of contact with the urogenital sinus.

Influence of Tfm in the heterozygous state

As indicated in Table 1 in experiments No. 3, No. 6 and No. 7, the mothers were heterozygous for Tfm (testicular feminization). Therefore, embryos with testes were either normal male (XY) or Tfm embryos (Tfm/Y). On day 15 of pregnancy, normal XY and Tfm embryos were distinguished by co-culture of testis and Wolffian body as described in Hannapel et al. (1981). On day 16 male embryos were discarded. On day 17, Tfm embryos were identified by direct inspection of the Wolffian body.

The Tfm mutation of the mouse is characterized by complete androgen insensitivity. Since Tfm is X linked, heterozygotes are X-inactivation mosaics with respect to androgen sensitivity. The mosaic of androgen-sensitive X+ cells and androgen-insensitive XTfm cells is, however, only expressed in androgen-dependent male development. Without testosterone both cell types follow constitutively the female pathway of development.

Experiment No. 6, Table 1, was especially designed to show the effects of the mosaic in the development of the genital tract. Only XX embryos with ovaries were used. Because of the segregation of the maternal X chromosomes, about half of the embryos were expected to be heterozygous for Tfm. Embryos with ovaries thus were either normal females or females heterozygous for Tfm. It was not possible to tell the Tfm heterozygotes in advance. Without testosterone genital tracts from Tfm heterozygotes develop as normal females. Therefore they were carried on as XX. Only in explants cultured with testosterone was there a chance to identify a Tfm heterozygote by the expression of the X-inactivation mosaic. The explants were examined according to the criteria for morphogenetic behaviour characteristic for development without or with testosterone as derived from the explants of experiments No. 7 and No. 13 (Table 1). After all in only one explant fixed on day 17, were there clear indications that we were dealing with a Tfm heterozygote. The explant is documented in the reconstruction of Fig. 8 and in the sections of Fig. 4. On the left hand Wolffian and Müllerian ducts fused with one another as in female development. However, as can be seen in the reconstruction of Fig. 8, fusion was restricted to a short segment of the ducts. Above and below this area and on the right side of the explant the ducts separated as in male development. This observation indicated that in the left Wolffian duct an area with androgen-insensitive cells was present in the epithelium or even more likely in the Wolffian mesenchyme. In this area, fusion with the Müllerian ducts could not be prevented by testosterone.

Since expression of the mosaic was observed in only one specimen, this finding cannot be generalized. Further experiments are in progress.
DISCUSSION

The nowadays generally accepted view of the development of the vagina is based on the work of Koff (1933). He described the development of the vagina in human embryos ranging from 11 mm to 169 mm. On the basis of graphic representations drawn to scale from serial sections and from wax-plate reconstructions, he concluded that the vaginal plate is formed by proliferation of two epithelial pockets arising from the sinus epithelium. He coined the term 'sinovaginal bulbs' which is now widely used in textbooks. According to the description of Koff (1933), the entire sinus vagina is derived from the sinovaginal bulbs and is only of sinus origin in so far as the bulbs evaginated from the sinus. His interpretation implies that the vaginal plate arises by upgrowth of the sinovaginal bulbs. On weighing the arguments, however, as done by O'Rahilly (1977) in his comprehensive review on the development of the human vagina, one realizes that other interpretations have not been definitely excluded. In particular, this holds true for the formerly widely held opinion that the vaginal plate grows down to the perineum and that the Wolffian ducts contribute to its formation.

The epithelial pockets which form the sinovaginal bulbs are continuous with the remnants of the Wolffian ducts. Therefore, the first investigators obviously took it for granted that the epithelial pockets were identical to the lower parts of the Wolffian ducts. Correspondingly, Hart (1901) called the structures 'Wolffian bulbs' and assumed, that the lower third of the vagina down to the hymen was of Wolffian duct origin. With our own observations in mind, statements such as that of Hart (1901) appear quite convincing: 'In two previous communications I discussed the questions of the origin of the hymen and vagina. I there attempted to show that the lower ends of the Wolffian ducts enter into the formation of the former, and that the latter was Müllerian in origin only in its upper two-thirds, the lower third being formed by blended urogenital sinus and Wolffian ducts' (p. 330). The quotation seems to indicate that the role of the Wolffian ducts in vagina development was a clear fact from the beginning. However this is not the case. A closer look at the evidence presented by Hart shows that it consists of a combination of scanty observations in human embryos with intelligent interpretations of adult female anatomy and far-stretched reasoning in comparative embryology by combining ideas on gastrulation and germ-layer theories with formation of the vagina of marsupials.

Mijsberg (1924) used the same terms ('Wolff'sche Bläschen' and 'Wolff'sche Höcker'). He was convinced that only a small segment of the vaginal anlage was of Wolffian origin located between the upper-Müllerian-derived and the lower-sinus-derived portions. In our eyes, his statement is also amazingly true: 'The meaning of the fusion of Wolffian and Müllerian ducts at their lower ends which in the human leads to the situation that the Müllerian ducts are connected to the sinus via the Wolffian ducts, is hard to understand. Also in other species a similar fusion occurs' (p. 746). With our knowledge on the endocrine regulation of sex
organ development, we are tempted to add: incorporation of Wolffian duct tissue in the vaginal plate makes sense in respect to hormonal regulation. It allows for the switch off of constitutive vaginal development in the male by simply inducing male development in the respective duct segments via testosterone.

In his monograph on the derivation and differentiation of the vaginal epithelium Forsberg (1963), in principle, followed Koff (1933). In his description of vaginal development in the mouse and other mammals he used the term ‘sinus protrusions’ instead of sinovaginal bulbs. However, in his concluding remarks on the histochemical characterization of the epithelium of Müllerian and Wolffian ducts and of the urogenital sinus in human embryos he states: ‘After taking into consideration the circumstances that may have influenced the enzyme activity in the different epithelia, it must be pointed out that the distribution of acid phosphatase, esterase, and arylsulphatase, possibly also leucine aminopeptidase, favours the view that the vaginal plate is a Wolffian derivate; particularly as the morphological investigations also suggest this. Another possibility is that the Wolffian epithelium induces a cranial growth of the sinus epithelium, thereby changing its character’ (p. 113).

From the above eclectic citations and even more from the comprehensive discussion of the topic by O’Rahilly (1977) it appears that the question of derivation of the vagina cannot be settled solely by classical methods of descriptive embryology. The same is true for the closely related controversy of upgrowth of sinovaginal bulbs versus downgrowth of Wolffian epithelium.

Our in vitro approach had the advantage that morphogenetic movements were directly visible in the living explants. Direct comparison of constitutive female and testosterone-induced male development allowed the unequivocal follow up of the fate of the structures in question.

The characteristic behaviour of Müllerian and Wolffian ducts during female or male development is visualized in Fig. 14, which is based on the reconstructions of the explants of day 19. The location of the ducts and their histological appearance in cultures without testosterone corresponded to the in vivo situation in the female mouse embryo as described by Forsberg (1963). However, our observations show that the ‘sinus protrusions’ of Forsberg are in fact identical to the lower segments of the Wolffian ducts.

Witschi (1970) assumed that in the human embryo the ‘sino-vaginal bulbs’ of Koff (1933) were identical to the lower segments of the Wolffian ducts. He re-examined the embryos used by Koff and concluded, that the vaginal plate arises by downgrowth behind the urogenital sinus, and that the Wolffian ducts take part in its formation. In the drawing of Koff’s 100.5 mm human embryo re-interpreted by Witschi and reproduced by O’Rahilly (fig. 2, p. 129 in O’Rahilly (1977)), the location and pattern of coalescence of Müllerian ducts and Wolffian ducts are identical to that exhibited by our day-19 specimen without testosterone (Fig. 14A). In his fig. 3 (p. 130) O’Rahilly (1977) compares as equivalent the ‘classical
Fig. 14. Female and male development drawn after the reconstructions of Figs 10 and 11. Without testosterone the Müllerian ducts elongate caudally and fuse with the Wolffian ducts. Thus the starting point for the vaginal plate is formed which elongates further by separation from the urogenital sinus. With testosterone fusion of Wolffian and Müllerian ducts is prevented by male differentiation of the respective Wolffian duct segments. The formation of a sinus vagina is thus inhibited.

View' of Koff assuming upgrowth of the vaginal plate with Witschi's view of vaginal downgrowth and incorporation of Wolffian ducts. Our experiment demonstrates that Witschi's view was correct.

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The contribution of G. Bok will be submitted as inaugural dissertation at the Fakultät Theoretische Medizin der Eberhard-Karls-Universität Tübingen.

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