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# Involution and hormone-induced persistence of the *M. sphincter (levator) ani* in female rats

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## INTRODUCTION

It has been assumed that in rats the levator ani muscle (Greene, 1935) exists only in the male (Hayes, 1965). Hayes concluded that this muscle is not homologous with the levator ani in primates, and the re-examination of morphological data has led us to agree with this conclusion, but not with his assumption that the muscle represents the dorsal part of the m. bulbocavernosus. An ontogenetic study showed that the term 'm. bulbocavernosus dorsalis', suggested by Hayes (1965) is not justified (Čihák, Gutmann & Hanzlíková, 1967).

The muscle develops from an originally uniform blastema showing homology with the m. sphincter cloacae of Monotremes and Marsupials. Gradually the m. ischiocavernosus separates from this blastema, whereas the rest of the blastema divides into a ventral, urogenital, and a dorsal, anal, part. The muscle in question develops from this dorsal part and is therefore homologous with the typical m. sphincter ani (externus). Thus the muscle of the male rat is, on the basis of its homology, and in accordance with descriptions in the older literature, a m. sphincter ani (Krause, 1884; Holl, 1896). Its insertion on the bulbus corporis cavernosi urethrae and to the coccygeal fascia are only secondary species modifications. Differentiation of the myoblasts occurs bilaterally and symmetrically, so that the loop consists of independent right and left halves. Separate branches from the pudendal nerves, two rows of endplates at the ventral border of the muscle and a connexion of the two sides by a fibrous Z-like raphé ventral to the caudal vertebrae demonstrate its bilaterality (Čihák *et al.* 1967).

Although the muscle persists only in adult male rats, the blastema from which it develops is present in the embryos of both sexes, and until the 18th d of embryonic life development is practically alike in both male and female. From this time until birth, however, the development of the muscle in female rats is considerably retarded and from 7 to 21 d of postnatal life complete involution of the muscle of the female rat occurs. This process, leading to sexual dimorphism, is not observed to such an extent elsewhere in the skeletal muscular system. Wasting of the m. sphincter ani after castration can be prevented by testosterone (Wainman & Shipounoff, 1941) and the increase in weight of this muscle after testosterone is widely used to assay the myotropic activity of androgenic steroids (Eisenberg & Gordan, 1950). For these reasons an

attempt was made to influence the process of involution of this muscle in female rats by application of testosterone before its involution was complete (Gutmann, Hanzlíková & Čihák, 1967).

This paper describes the postnatal development and fate of the muscle in (*a*) male rats, showing the typical development from myotubes to muscle fibres; (*b*) female rats, showing involution of the muscle and (*c*) female rats, in which persistence of the muscle was achieved by perinatal application of testosterone.

## MATERIAL AND METHODS

The m. sphincter ani of 93 rats of both sexes at the ages of birth, 1, 3, 5, 7, 10, 14, 21, 30, 60 and 120 d were examined. Absolute and relative (i.e. muscle weight as % O body weight) weights of dissected muscles were determined for male rats and for female rats which had received 1 mg testosterone propionate subcutaneously from the day of birth until death. In one group of female rats application of testosterone was interrupted after 1 month and the weights were determined 1 to 4 months later.

Microscopical, histological and electron microscopical examinations were carried out on the specimens, including a group of animals in which testosterone ($4 \times 0{\cdot}5$ mg) had been given subcutaneously to the mothers during the last week of gestation. For microscopical examination the muscle was dissected under a binocular microscope. Materials for examination by optical microscopy was fixed in Bouin or 10 % neutral formalin, embedded in paraffin and sectioned serially in a plane transverse to the spine and rectum. The sections were stained with Weigert's haematoxylin and eosin.

For electron microscopy, the muscles were quickly exposed, dissected free from the other perineal muscles and fixed *in situ* by immersion in a 2·5 % solution of glutaraldehyde buffered to pH 7·4 in Millonig's buffer (Sabatini, Bensch & Barnett, 1963), postfixed in 1 % $OsO_4$ and embedded in Vestopal. The sections were stained with uranyl-acetate and lead-citrate (Reynolds, 1963) and examined with a Tesla BS 413 electron microscope.

## RESULTS

### (*a*) *Male rats*

In normal postnatal development m. sphincter ani of the male rat shows progressive thickening and differentiation of the muscle fibres. In the newborn (Fig. 1) the muscle, composed of myotubes, is slender and shows a proximal ventral thickening. Longitudinal myofibrils are discernible, but cross-striations cannot be distinguished with the light microscope until 1 d after birth. The 3-d-old muscle is practically the same size, cross striation of the myofibrils is clearer and there is a change of myotubes into muscle fibres with the characteristic shifting of the nuclei to the periphery. Dorsally, where the raphé is being formed, typical myotubes are still present. By 7 d all the myotubes have differentiated into muscle fibres; the epimysium is now clearly outlined and the surrounding mesenchymal tissue has been transformed into fat. The muscle has enlarged and the fibrous raphé can now easily be identified from the muscle fibres. The muscle fibres of 10 d rats are thinner than those of the neighbouring muscles such as erector spinae; average diameter of the fibres in m. erector spinae is 12 $\mu$m, while the average diameter of the m. sphincter fibres is 7 $\mu$m.

Development of the muscle is complete at 14 d; a well-differentiated perimysium

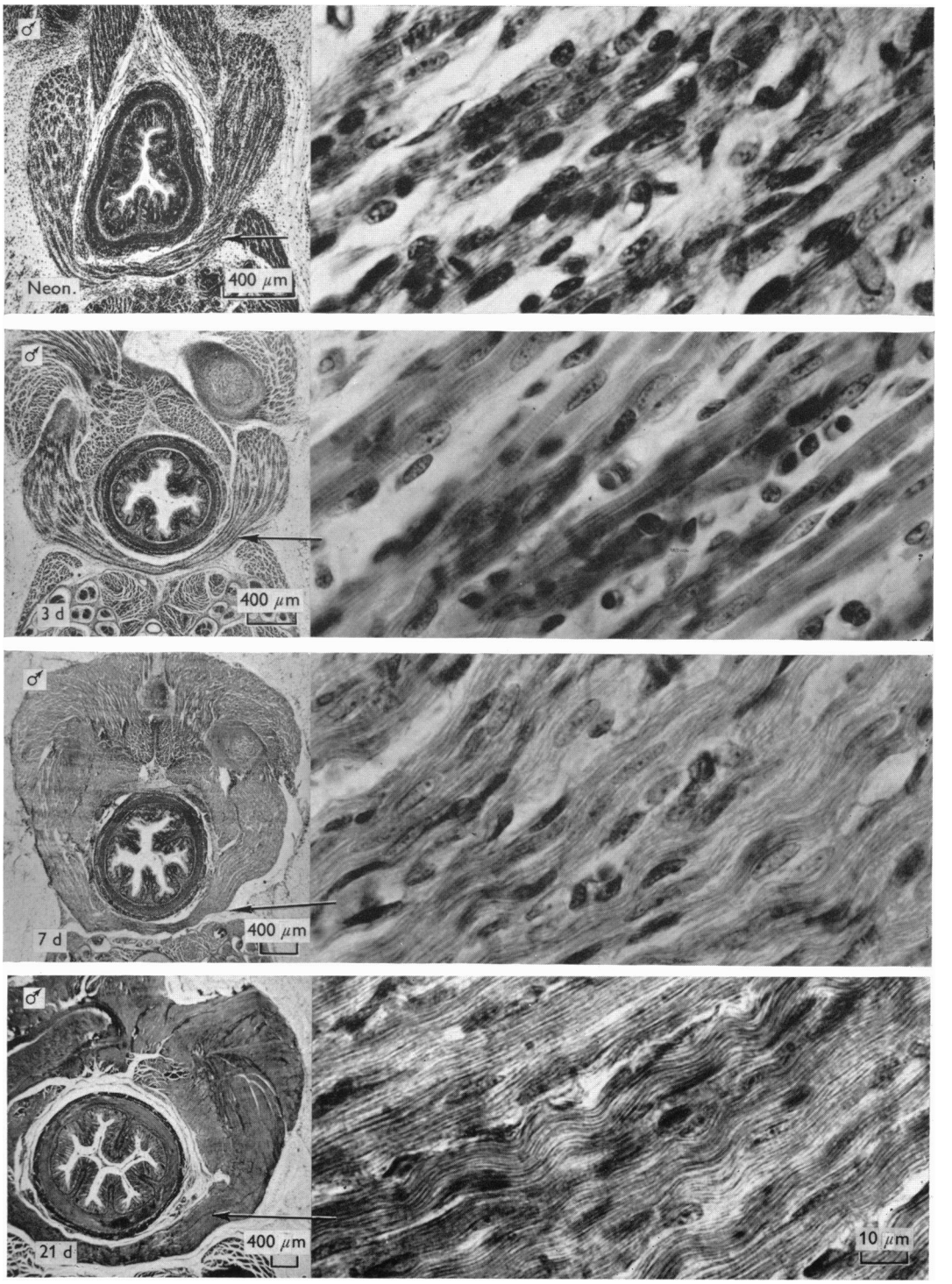
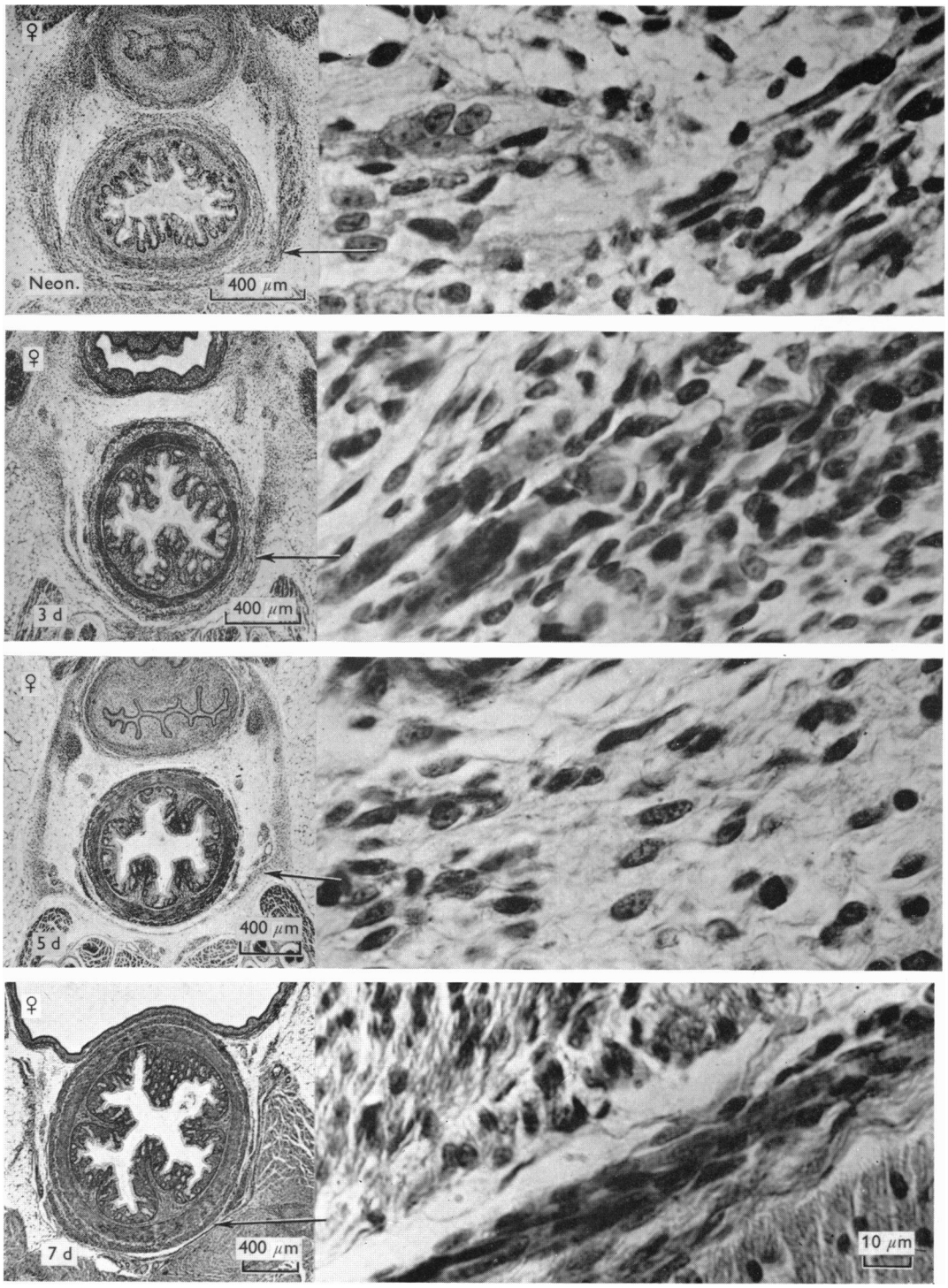



Fig. 1. Progressive development of the m. sphincter (levator) ani in male rats. The subsequent figures demonstrate growth and differentiation of the muscle in newborn rats and in rats 3–21 d after birth. Arrows indicate the location of the higher power photograph.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is now present, although the muscle fibres are still thinner than those of the neighbouring muscles. The retardation of differentiation and growth of the muscle fibres as compared with other skeletal muscles is no longer observed in 21 d male rats.

(*b*) *Female rats*

The m. sphincter ani of the female develops similarly to that of the male until the 18th d of embryonic life. Myotubes differentiate from the blastema at the same time as involution is occurring. In some animals this involution is rapid and there is no transformation into muscle fibres, while in the others the involution is slower, and this allows the myotubes to differentiate into typical muscle fibres.

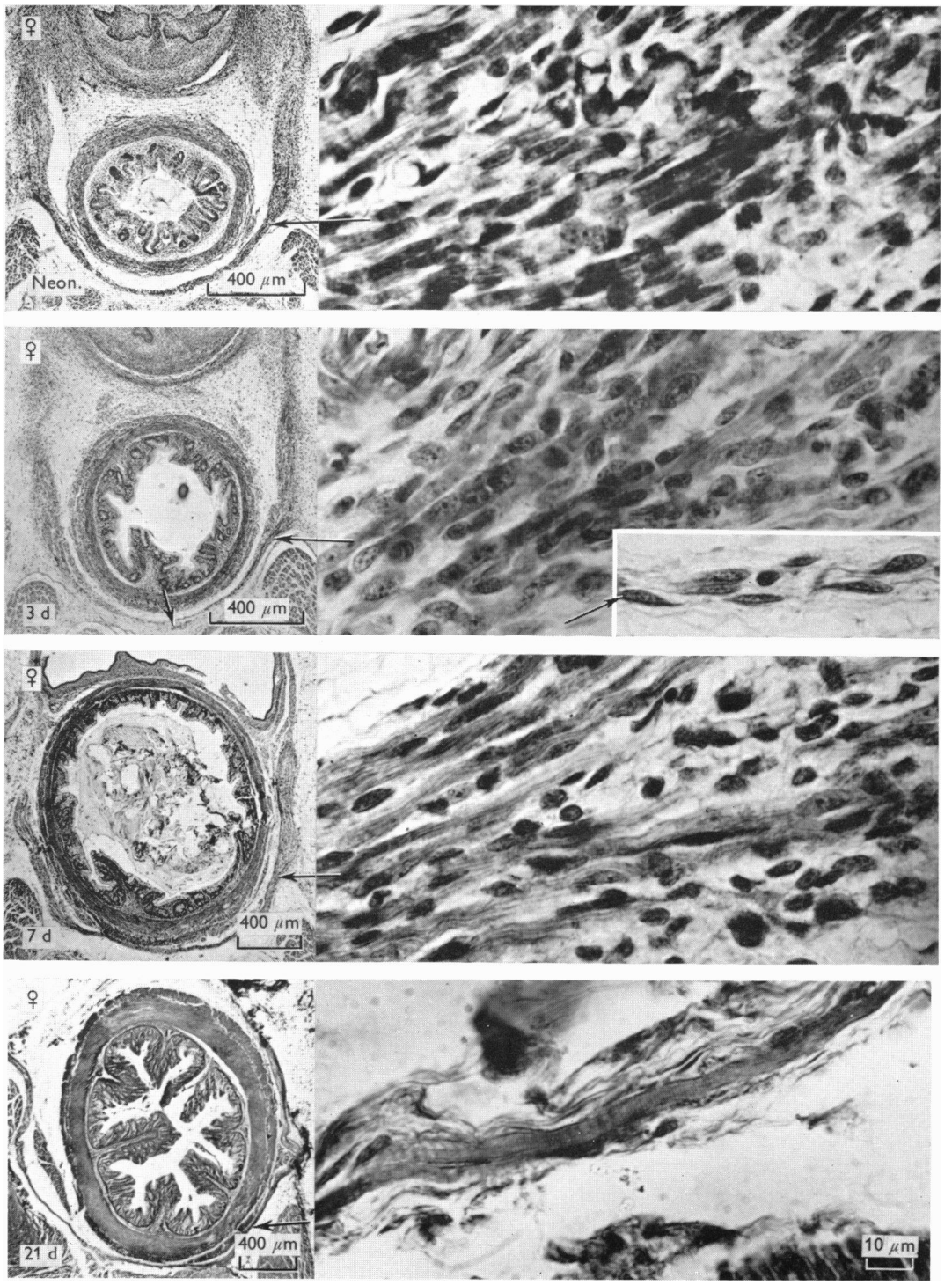
(1) *Female rats with rapid destruction of the m. sphincter ani*

In this group the muscle shows regressive changes at birth (Fig. 2). In the newborn female rat the few, thin myotubes contain very few myofibrils, and these show no cross-striation. Regressive changes are seen in the myoblasts and myotubes as pycnotic nuclei, and occasional eosinophilic fragments of cytoplasm. The muscle loop encircling the rectum is interrupted and from the ends of the remaining myotubes regenerating strands of cytoplasm with rows of nuclei and occasional stumps of myofibrils can be seen (Fig. 2). Abortive regeneration is evidently accompanying muscle destruction. On the 3rd d of rapid involution only strands of pycnotic nuclei and nuclei of the fibroblasts remain, as most of the myotubes have been destroyed. No differentiation of muscle fibres has taken place (Fig. 2). On the 5th d of postnatal development the site of the muscle is outlined only by undifferentiated mesenchymal cells, their arrangement still suggesting the original shape of the muscle (Fig. 2). On the 7th and 10th d after birth only a thin fibrous strand is seen, the surrounding mesenchymal tissue being transformed into fatty tissue. Occasionally in these strands fragments of muscle fibres with rudimentary cross-striations are found (Fig. 2).

(2) *Female rats with slow destruction of the m. sphincter ani*

In this group of female rats the two processes of differentiation and involution take place at the same time. At birth the muscle is composed of thin myotubes (diameter 2–3 $\mu$m) which undergo a partial destruction. There are, however, few eosinophil rests of the myotubes. The majority of the nuclei in the thin myotubes are clearly outlined with normal chromatin; some pycnotic nuclei and abortive regeneration can be seen. The myofibrils are cross-striated (Fig. 3). Mesenchymal tissue separates the myotubes and there is no sign of fascial formation; the myotubes mostly do not reach the midline. A characteristic feature of the muscle in the female is that the enlarged proximal part, which in male rats inserts on to the bulbus penis, has no well-defined insertion and lies free in the mesenchymal tissue encircling the urogenital orifice. Instead of the typical raphé the two muscle bands are joined by a thin strand of connective tissue. In the 3 d animals the muscle consists of myotubes with nuclei and

Fig. 2. Regressive changes of the m. sphincter ani in female rats. Rapid regression of the muscles at birth is shown by poorly developed myotubes with abortive regeneration in newborn rats and continues up to 7 d resulting in formation of fibrous strands intermingled with traces of cross-striated cells.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myofibrils as at 1 d and the dorsal connecting part of the two muscles is similarly a thin strand of connective tissue consisting of one to two rows of loosely connected fibroblasts (Fig. 3).

At 7 d, although the muscle is reduced in size, the myotubes have differentiated into thin cross-striated muscle fibres (Fig. 3). Differentiation proceeds as in the male animals in the proximal enlarged parts of the muscle, but dorsally myotubes are still present. In some animals the myotubes reach the midline; the dorsal part however remains thin, consisting of one to three rows of myotubes, and cannot be found on microscopical examination. The muscle is reduced in size but continues to differentiate. Its proximal fusiform part has become thinner, the diameter of the muscle fibres being 3–3·5 $\mu$m. Fourteen days after birth the muscle is even thinner; the fibres are well differentiated but no thicker than 3·5 $\mu$m. Connective tissue now starts to dominate the picture. In animals aged 21 d (Fig. 3) involution is almost complete, and the muscle consists of only a fibrous strand with occasional remnants of muscle fibres.

(*c*) *Testosterone treated female rats*

In one group of animals testosterone (4 × 0·5 mg) was given subcutaneously to the mothers during the last week of gestation. The muscles of the newborn females from these mothers had the size and form of those in newborn male rats, showing well-differentiated myotubes with cross-striated myofibrils. The proximal part of the muscle was thick and the site of the future raphé well defined (Fig. 4). In another experiment testosterone (1 mg) was applied twice weekly to newborn female rats. The muscles of these animals had the same size and shape as that of 3-d-old male rats. The proximal parts of the muscle were thick and the loop around the rectum showed the well defined raphé. The myotubes had started to differentiate into muscle fibres. The surrounding mesenchyme was freely continuous with that between the muscle fibres (Fig. 4). The muscle of 7 d animals had continued to grow, but it was more slender and well developed muscle fibres were fewer than in the male rat. The muscle fibre diameter was 5–7 $\mu$m, and the myofibrils showed clear cross-striation (Fig. 4). The dorsal part of the muscle was well developed, so that raphé could easily be identified. In muscles of some animals destruction of a few muscle fibres with regeneration from the ventral part of the muscle had occurred.

Later growth of the muscle continued, as even in 10-d-old animals no sign of destruction or involution of muscle could be found, and the configuration was the same as in male rats. This progress was maintained in the 14-d-old animals, where the diameter of the muscle fibres was 7–12 $\mu$m. The relative weight of the muscle was maintained in 21-d-old animals, when it showed the mature histological structure with fibre diameter of 10–12 $\mu$m. Hence testosterone induced proportional growth, shape and differentiation of the m. sphincter ani, so that growth and differentiation proceed as in the male.

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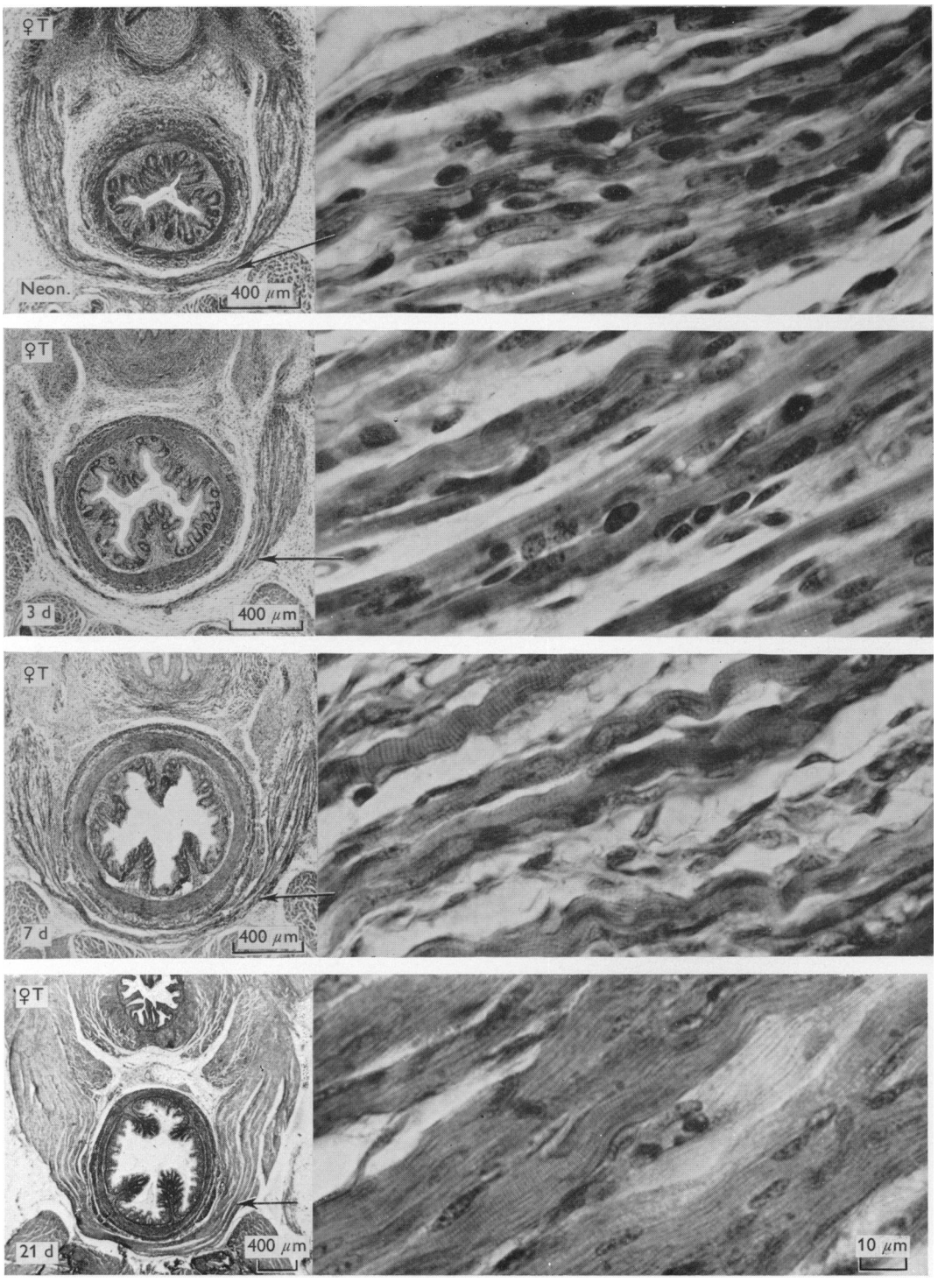
Fig. 3. Regressive changes of the m. sphincter ani in female rats. Relatively slow regression of the muscle permits differentiation of myotubes to muscle fibres, total mass of the muscle decreasing to strands of fibrous tissue with single muscle fibres until 21 d after birth. The dorsal portion of the muscle is fibrous from the 3rd postnatal day (arrows).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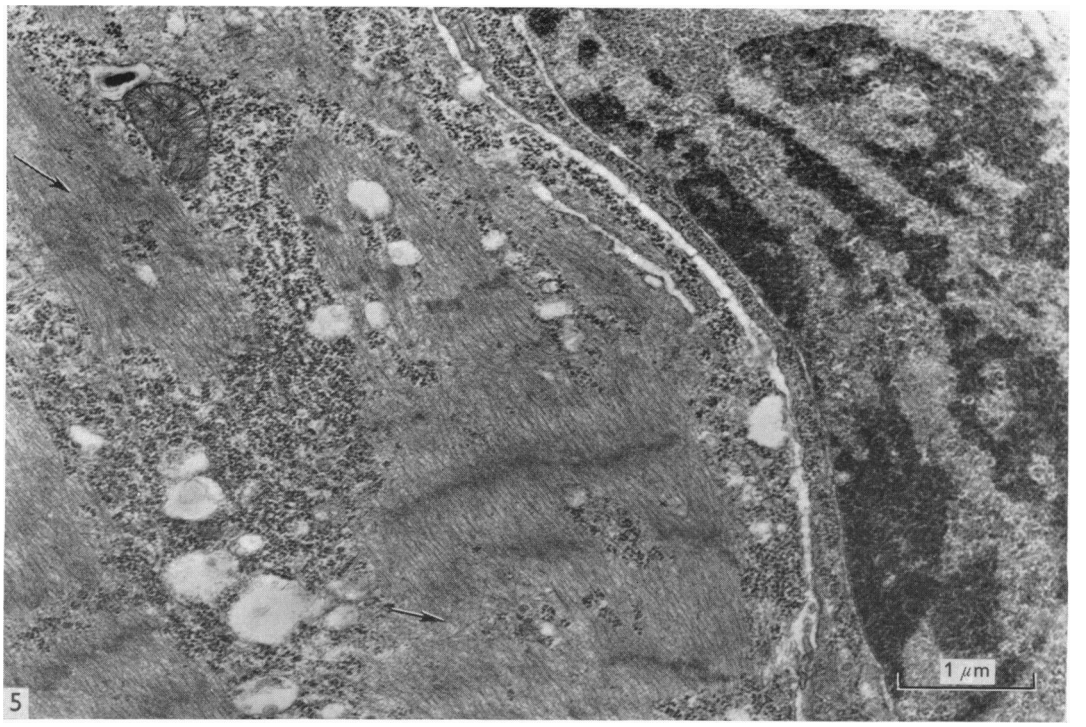
(*d*) *Ultrastructural changes*

The electron microscopic investigation of the m. sphincter ani of female rats undergoing postnatal involution is difficult. The extremely thin muscle loop has to be prepared by isolation and dissection for *in situ* fixation, and often the dissected muscle proved to be only a strand of fibroblasts. Figure 5 shows a longitudinal section through the muscle of a 3-d-old female animal. The process of involution is shown by the irregular breakdown of myofibrils. The myofilaments are irregularly orientated and their regular alignment is lost. It is difficult to outline individual myofibrils, as myofilaments follow irregular courses or are disposed in a whorl. In some places the process of myofibrillar degeneration leaves dedifferentiated sarcoplasm, with rests of irregularly orientated myofilaments, vacuoles and accumulation of glycogen granules. Although the Z lines are still visible, they lose their normal outline and at some places break down, with dissolution of the Z line material. Vacuolar degeneration appears to be a typical feature; some of the vacuoles are filled with membranous circular structures which have also been described in denervated muscle and could be the remains of degenerate mitochondria (Fig. 6) (Lee, 1963). The muscle nucleus may show deep invagination and karyolysis takes place with focal disruption of the nuclear membrane (Fig. 7). There are relatively few ribosomes in the nucleolus which is filled with irregular patches of coarsely granular chromatin. Condensation of chromatin at the periphery of the nucleus and an annular type of nucleolus are often found. Thus involution is characterized by the breakdown of myofibrils, vacuolar degeneration, accumulation of glycogen granules and degenerative nuclear changes. This corresponds to the light microscopy findings of 'fragmented myotubes', loss of cross-striations, still visible myotubes containing few myofibrils without cross-striation and eosinophilic cellular fragments.

The muscle of 3-d-old male rats contrasts considerably with the involuting female muscle (Fig. 8). The myofibrils in all parts of the muscle are thin but the sarcomeres show a normal pattern of cross-striation. The Z line is often irregular, but this might be a technical artefact. There are many ribosomes and glycogen granules near the sarcoplasmic reticulum but larger irregular clusters of glycogen are not seen. The sarcoplasmic reticulum is well outlined, but triads are not yet discernible. Cross section of the muscle shows that development of the myofibrils has proceeded normally (Fig. 9) to fill most of the fibre. The muscle of the 46-d-old male animal shows the normal pattern of a fast muscle with M lines and triads.

The persisting muscle of a 3-d-old female rat receiving testosterone has similar structure to that of 3-d-old males. However, the muscle shows more irregular myofilaments, more clusters of glycogen, some vacuoles and lysosomal structures and pinocytotic activity of the sarcolemma is more pronounced. The nucleus shows irregular clusters of chromatin, but the nuclear membrane is well defined (Fig. 10). Longitudinal sections through the persisting muscle at this time show well outlined myofibrils with the normal pattern of cross-striation. The sarcoplasmic reticulum

Fig. 4. Progressive development of the m. sphincter ani in female rats treated with testosterone. The subsequent figures demonstrate an analogous development, growth and differentiation as in muscles in male rats.

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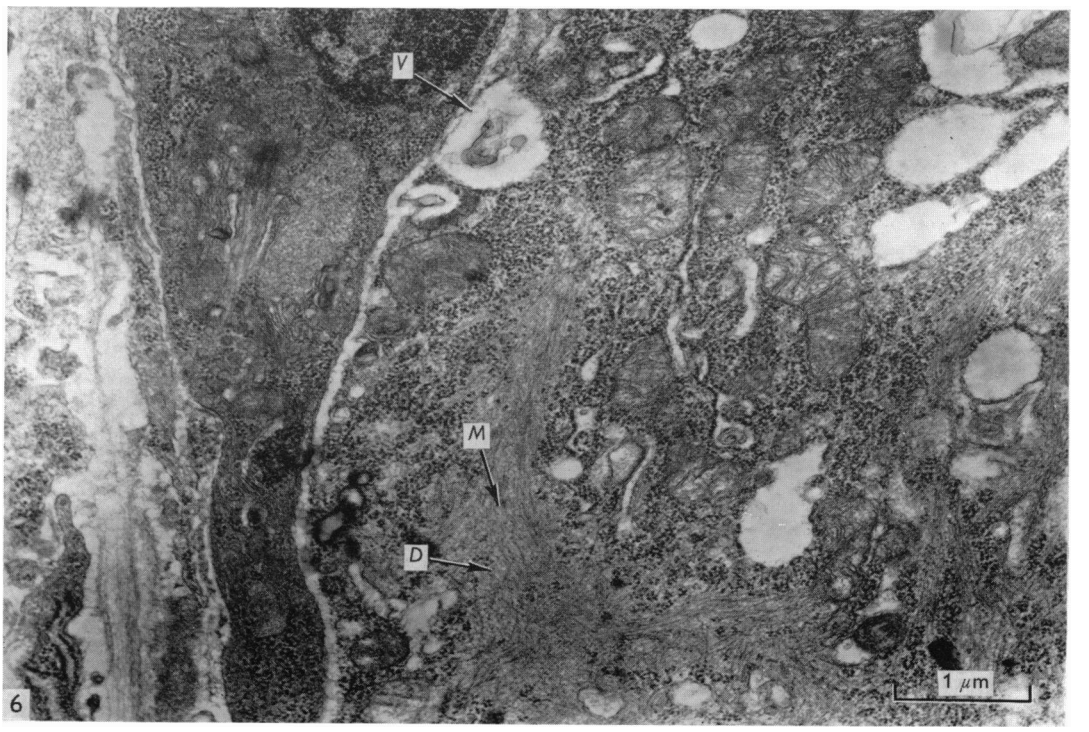


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appears to be enlarged, and the number of mitochondria to be increased (Fig. 11). The muscle of 47 d animals shows the normal pattern of cross-striation (Fig. 12). Thus, testosterone treatment of female rats preserves an ultrastructure similar to the normal male for up to 2 months.

*(e) Weight changes in the m. sphincter ani of male rats and of female rats treated with testosterone*

Table 1 shows that the weight of the m. sphincter ani of female rats treated with testosterone for 1 month is practically the same as that of the 1-month-old male rats. After 2 months the weight is below that of the male and the difference increases in the third month, as the muscle of the treated female is now only 50 % of the weight of the 3-month-old male rats. A similar relationship holds at 4 months, thus the main retardation of growth occurs during the third month of development even in the presence of androgens. Figure 13 shows these relations and also demonstrates that cessation of treatment leads immediately to retardation of growth of the muscle. One month's treatment is thus not sufficient to ensure further growth, and high levels of testosterone in the blood are apparently necessary to assure continued growth.

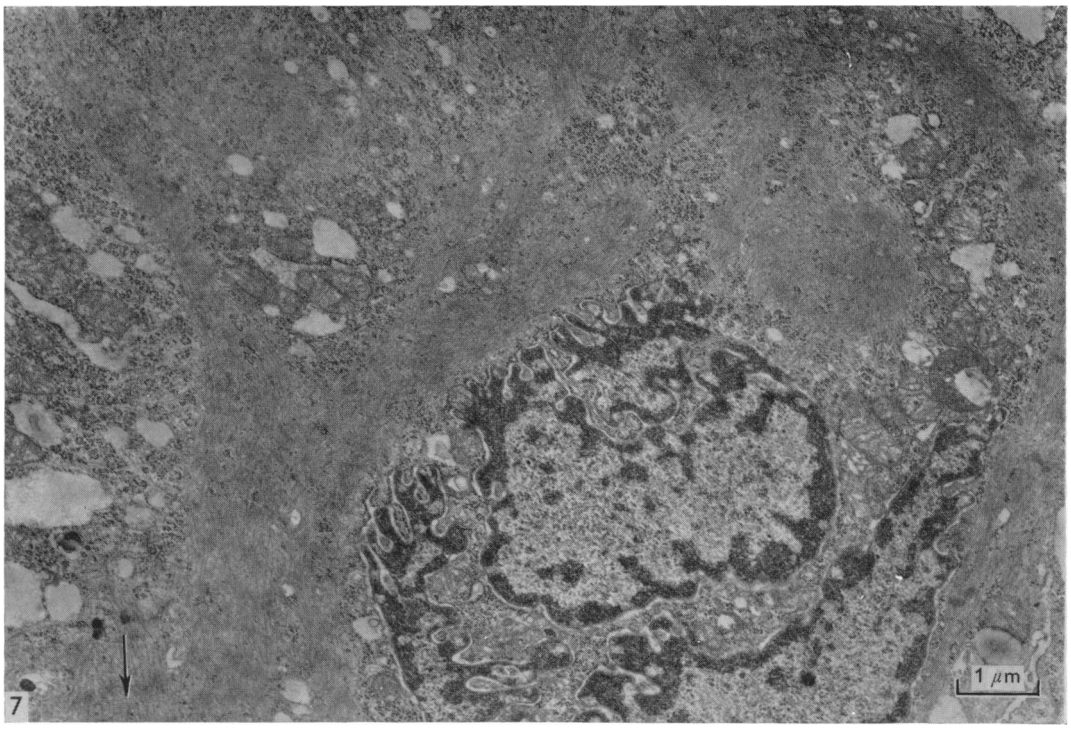
Table 1. *Absolute and relative weights of the MSA of* 1- *to 4-month-old rats (weight of muscle expressed as body weight* $\times 10^{-3}$)

(The female rats received 1 mg testosterone propionate (TP) twice weekly, beginning with the day after birth.)

| | Female treated rats (MSA) | | Male non-treated rats (MSA) | |
|---|---|---|---|---|
| Type of expt. | Absolute weight (mg) | Relative weight | Absolute weight (mg) | Relative weight |
| Age 1 month | | | | |
| 1 month TP treatment ($n$ = 6) | 19·6±1·9 | 0·201 | 19·7±1·4 | 0·210 |
| Age 2 months | | | | |
| 2 months TP treatment ($n$ = 6) | 68·0±10·6 | 0·321 | 97·5±9·4 | 0·541 |
| Age 3 months | | | | |
| 3 months TP treatment ($n$ = 5) | 80·2±6·9 | 0·316 | 182±19·5 | 0·644 |
| Age 4 months | | | | |
| 4 months TP treatment ($n$ = 5) | 87·2±3·0 | 0·286 | 230·6±12·6 | 0·650 |
| Age 2 months | | | | |
| 1 month TP treatment, with subsequent cessation ($n$ = 5) | 38·6±4·5 | 0·216 | 97·5±9·4 | 0·541 |

Fig. 5. Longitudinal section of the sphincter ani muscle of 3 d female rats. Note irregular course of the thin myofilaments (upper arrow) with loss of normal alignment of myofibrils, irregularities and dissolution (lower arrow) of Z lines. Vacuolar degeneration and accumulation of coarse glycogen granules.

Fig. 6. As Fig. 5. This shows advanced degeneration of muscle fibres, severe vacuolar degeneration with some vacuoles containing dense membrane remnants below the sarcolemma (*V*). Myofilaments are disordered, complete disarrangement of myofibrils with some myofilaments disposed in a whorl (*M*). Denser bodies may be derived from Z line material (*D*). Sarcolemmal invaginations penetrating at some places into dedifferentiated sarcoplasm.

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*(f) Contraction properties of the hormone-induced m. sphincter ani*

Contraction properties of the persisting m. sphincter ani of 5 female rats after 1 month's treatment with testosterone were similar to those of 1-month-old male rats. The data of the latter are in parentheses. After 1 month's treatment, weights of the persisting muscles are similar to those of the male control animals (Table 1). Maximal twitch tension was $3{\cdot}0 \pm 0{\cdot}5$ g ($3{\cdot}5 \pm 0{\cdot}30$), maximal tetanus tension $7{\cdot}5 \pm 2{\cdot}0$ ($9{\cdot}0 \pm 1{\cdot}2$ g). Fusion frequency was 60–90/sec. Contraction time (time to peak) was $41{\cdot}25 \pm 2{\cdot}24$ msec ($46{\cdot}0 \pm 2{\cdot}71$ msec), half relaxation time $28{\cdot}7 \pm 2{\cdot}24$ msec ($33{\cdot}0 \pm 0{\cdot}90$ msec). The persisting muscles are fast phasic muscles as are the muscles of 1-month-old control animals (Čihák *et al.* 1967). They do not react to acetylcholine or caffeine with a contracture.

## DISCUSSION

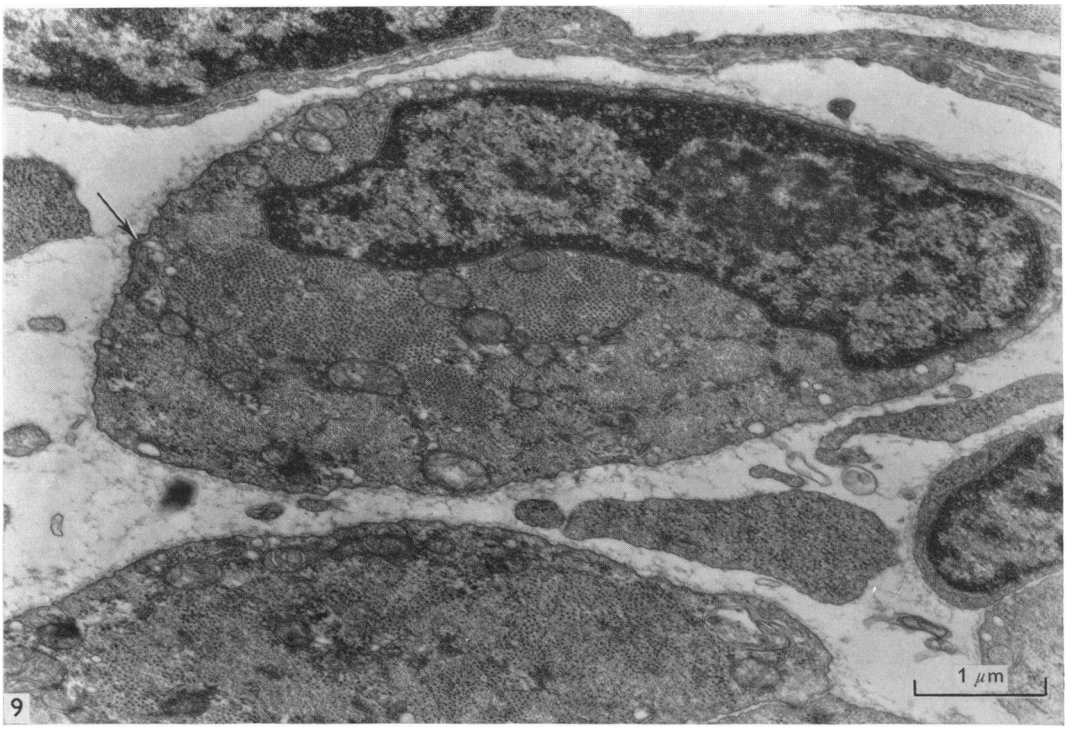
The histology of the m. sphincter ani (so called levator ani) has been studied in detail by Venable (1966). The muscle has the features of a fast ('white') muscle and to this correspond our measurements of speed of contraction. Venable (1966) suggested that the muscle fibres of both sides are continuous; however, a central raphé and two rows of endplates are present, demonstrating the bilaterality of structure.

The involution of the m. sphincter ani (so called levator ani) is not the only example of an orderly pattern of 'cell death'. Involution in the sense of 'cell death' occurs in fact during normal development (see Glucksmann, 1951). The resorption of the tail and opercular epidermis in tadpoles (Weber, 1957) and of the right paramesonephric duct of female chick embryos (Brachet, Decroly-Briers & Hoyez, 1958) have been described. Interesting examples are shown in the regressive changes during metamorphosis of insects, two different types of regression being distinguished, one of them occurring in the imago, the other starting at the beginning of each larval instar (Novák, 1967). An important example is afforded by the degeneration of the intersegmental muscles of the abdomen in the silk moth which occurs within 48 h after the ecdysis of the moth from the old pupal cuticle (Lockshin & Williams, 1965 *a*, *b*).

The involution of the sphincter ani is another example of 'programmed cell death' and leads to complete sexual dimorphism. No doubt the absence of the male hormones is the decisive factor for involution. It is well known that the development and differentiation of the genital tract at the time of delivery is not fully completed (Wiesner, 1934, 1935). It was deduced (Wiesner, 1935; Jost, 1947) that the gonads of male foetuses are primarily the source of an active influence on sexual differentiation, the absence of which would lead also to a loss of accessory organs, such as involution of the sphincter ani in the rat. The involution which appears to be closely connected to changes in the interplay of hormones could still be triggered off by other

Fig. 7. As Fig. 5. Complete disruption of normal alignment of myofibrils and shadows of previous Z lines (arrow). Disruption and invaginations of the nuclear membrane with annular condensation of chromatin at periphery.

Fig. 8. Longitudinal section of the sphincter ani muscle of 3 d male rat. Well developed pattern of cross-striation with some irregularity of the Z lines. Glycogen granules distributed in proximity of the sarcoplasmic reticulum.

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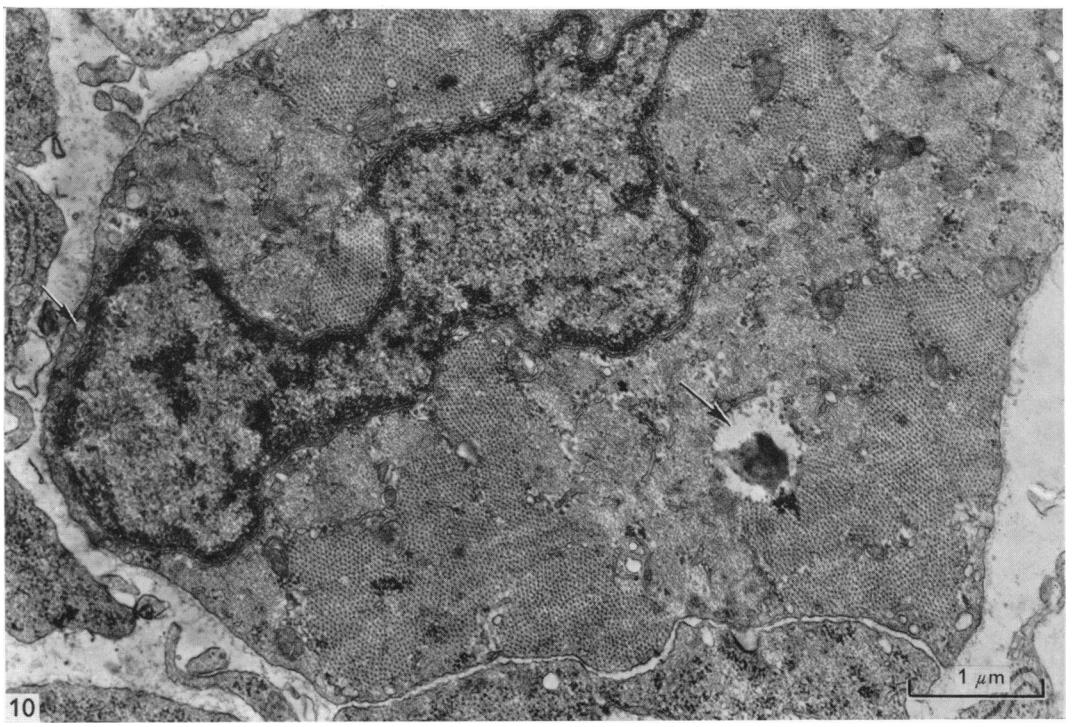


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mechanisms. Breakdown of the intersegmental muscles of the silk moth is potentiated by ecdysone; however, the breakdown of the muscles seems to be triggered by a neural mechanism and cessation of efferent impulses to the muscles was correlated with the process of involution (Lockshin & Williams, 1964). Complementary data of such a triggering mechanism would be difficult to obtain in our material. Further studies on the innervation of the m. sphincter ani in female rats should be rewarding.

The histological findings do not give clues for the mechanism triggering off the process of involution. It is of interest that application of testosterone will maintain the muscle even when denervation is performed perinatally (Hanzlíková & Gutmann, 1968). This finding suggests an absolute dependence of this muscle on hormonal level at least in the early stages of development.

The persisting muscle should provide a good model for studies of hormonal influences on muscle structure and metabolism. It should also be interesting to define central factors. Testosterone secreted from the interstitial cells maintains accessory sex organs, such as the seminal vesicles and the prostate gland, and this maintenance process applies apparently also to the m. sphincter ani. However, both the seminiferous tubules and interstitial cells are under the control of the gonadotropic hormones produced by the adenohypophysis (Lacy, 1967). Thus elucidation of the mechanisms controlling the process of involution will be difficult. However, the accessory sex tissues, including the m. sphincter ani, of the rat, are completely dependent on androgenic steroids for growth and function (Kochakian, Hill & Harrison, 1964). The increase in weight of the m. sphincter ani of castrated male animals after testosterone has been widely used as an assay method for the myotropic activity of androgens (Eisenberg & Gordan, 1950). However, the use of the muscle as a test for anabolic activity in castrated male animals has been criticized (Ahrén, Arvill & Hjalmarson, 1962), and the lack of a standard method comparing the relations of myotropic and androgenic effects of anabolic steroids has made therapeutic applications difficult (Krüskemper, 1965). The persistence of the m. sphincter ani in female rats may provide new methods of evaluation for the androgenic and myotropic actions of anabolic steroids (Gutmann *et al.* 1967).

SUMMARY

The m. sphincter (levator) ani, previously described only in male rats, has a blastema in both sexes. Its involution can be prevented by pre- or perinatal administration of testosterone. The postnatal development and fate of this muscle is described in male rats showing normal development from myotubes to muscle fibres, in female rats with involution and in female rats in which involution is inhibited by androgens.

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Fig. 9. Cross section of muscle fibre of the sphincter ani muscle of a 3 d male rat. Normal pattern of filaments at level of A and I bands. Increased pinocytotic activity below sarcolemma (arrow).

Fig. 10. Cross section of muscle fibre of the sphincter ani muscle of a 3 d female rat treated with testosterone. Advanced synthesis of myofilaments with normal pattern of filaments in A and I bands. Occasional fat body filled with dense material (arrow). Pinocytotic activity below sarcolemma (arrow).


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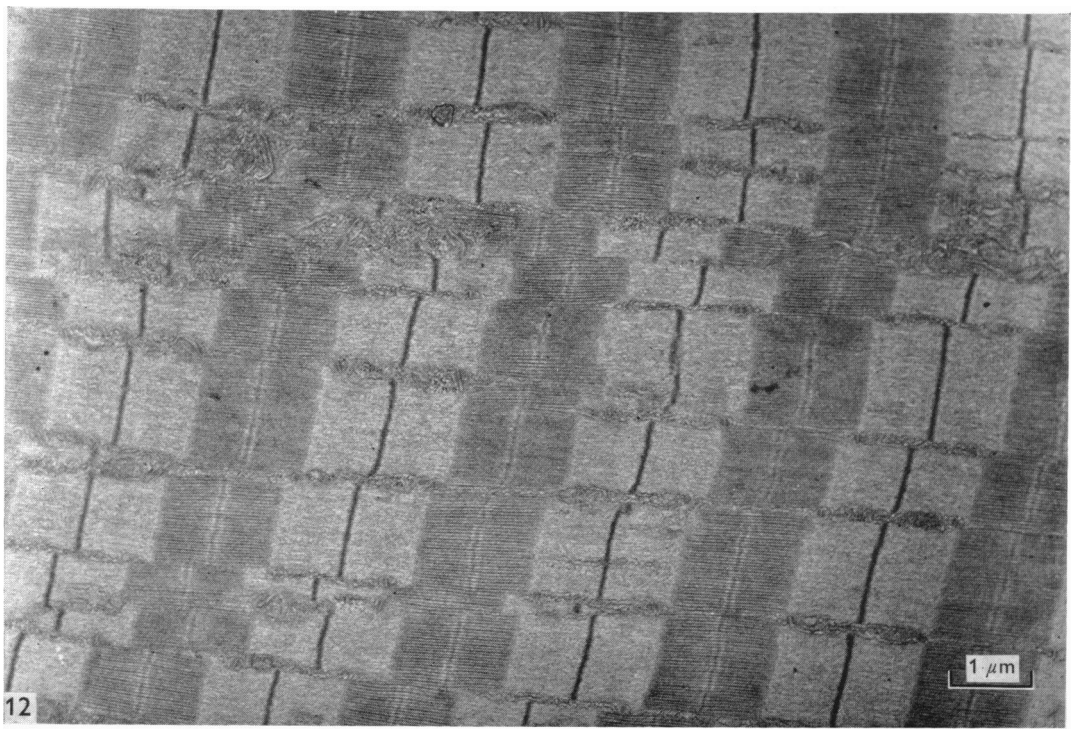


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Development of the muscle in male rats is completed between 14–21 d after birth, but its differentiation is retarded with respect to other skeletal muscles. In female rats the muscle undergoes either rapid regressive changes with abortive regeneration and replacement by a thin fibrous band about 7 d after birth or a slower involution lasting up to 3 weeks. Degeneration, with disarrangement and loss of myofilaments is evident 3 d after birth.

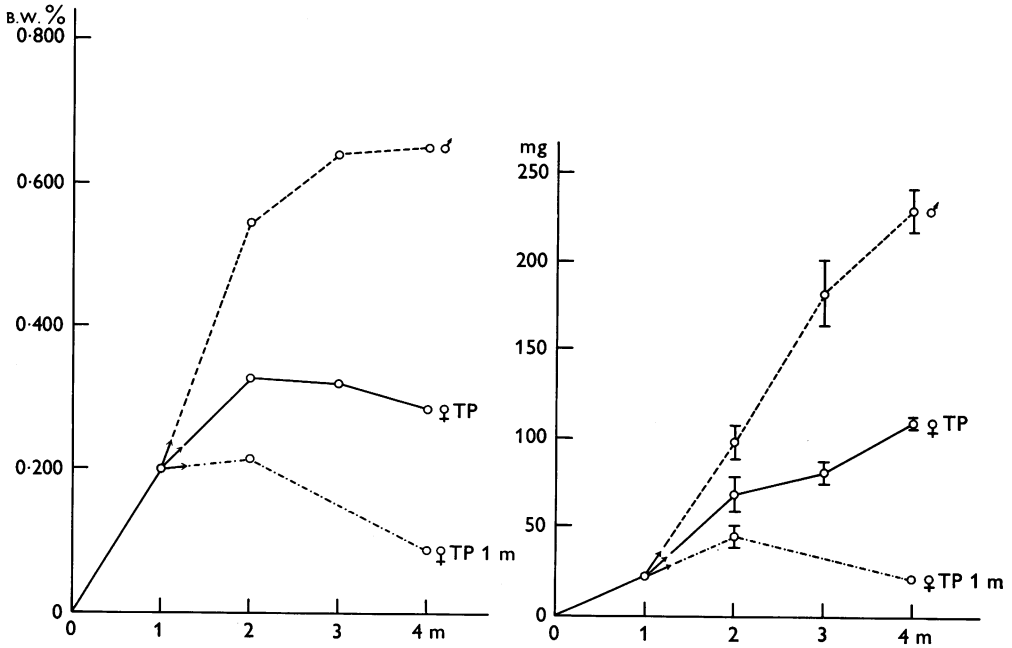


Fig. 13. Absolute and relative changes of the weights of the m. sphincter ani during 4 months after birth in muscles of male rats (♂), of female rats treated continuously with testosterone propionate twice weekly (♀ TP) and of female rats in which treatment was interrupted after one month (♀ TP 1 m).

After testosterone treatment, the cross-striated muscle of female rats develops almost normally until 1–2 months after birth. The contraction properties of the persisting muscle are similar at this time to those of the muscles of male rats. Interruption of the testosterone treatment leads immediately to weight loss of the muscle. The involution of muscle is an example of 'programmed cell death' leading to complete sexual dimorphism. The mechanisms controlling involution of the muscle in female rats are discussed.

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Fig. 11. Longitudinal section of the sphincter ani muscle of 3 d female rat treated with testosterone. Well developed pattern of cross-striation with myofilaments well outlined. Well developed but irregularly outlined longitudinal sarcoplasmic reticulum. Increased number of mitochondria.

Fig. 12. Longitudinal section of the sphincter ani muscle of 47 d female rat, treated continuously with testosterone. Normal pattern of cross-striation with well orientated sarcoplasmic reticulum.

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