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# The effects of denervation on skeletal muscle blood vessels (rat cremaster)

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Few reports have been published on the effects of denervation of skeletal muscle on the sensitivity of its vessels to various stimuli and the results are in part conflicting. The recently described method for directly observing the blood vessels in the rat cremaster (Grant, 1964) provided an opportunity for further investigating the matter.

The available information about the cremaster and its nerve supply proved insufficient, and further study was required before the vessels could be satisfactorily denervated. Then, at various times after section of the nerves supplying the cremaster, the responses of its vessels were tested to both topical and intravenous administration of vasoactive substances. Histological examination showed that total denervation leaves the vascular responses to constrictor and dilator substances apparently unchanged.

## METHODS

*Observation of the cremaster* during life was carried out as already described (Grant, 1964). By making the scrotal incision transverse, both cremasters may be exposed at the same time, thus allowing comparison of normal and denervated muscles. As a precaution, to prevent voided urine from spilling into the enlarged pool, the penis is pulled to one side by a stitch through the foreskin.

In exposing the cremaster, a convenient method for fixing the skin stitches is to surround the scrotum by a collar of perspex tubing, 25 mm deep and about 50 mm diameter which has a 30 mm gap to admit the scrotum. A notch is cut on the lower surface of the opposite side to accommodate the tail, the cable of the thermal junction in the anus and the stitch through the hind pole of the testis. The threads of the scrotal stitches are carried up over the perspex collar and then down to be fixed by pins to the cork board on which the rat lies. It is thus easy to adjust the direction and tension of the pull on the scrotum for the formation of the pool bathing the cremaster.

*The vasoactive substances* applied to the cremaster are adrenaline acid tartrate, noradrenaline bitartrate, acetylcholine perchlorate, histamine acid phosphate, 5-hydroxytryptamine creatine sulphate and succinylcholine chloride. Solutions were prepared as previously described (Grant, 1964). Concentrations stated in the text are in terms of the bases except for succinylchloride chloride.

*Surgical operations* were carried out under general anaesthesia with aseptic precautions. When recovery was intended, sodium pentobarbitone (3 mg/100 g rat) was injected intraperitoneally; otherwise urethane (150 mg/100 g) was used.

*Lumbar sympathectomy* was performed as described by Farris & Griffith (1962) except that the abdomen was opened by a left flank incision extended medially and

caudally to the symphysis pubis. This avoids pulling out the intestines which can be retracted to one side within the abdomen. The sympathetic chains were removed from just below the diaphragm to as low in the pelvis as could be reached. A rise of tail temperature at operation not associated with a rise of colon temperature and failure of tail temperature to rise when the body was warmed a few days later were taken as indices of a successful sympathectomy (Grant, 1963).

*Denervation of cremaster.* The same incision served to expose the four nerves required to be cut to denervate the left cremaster muscle (easier on that side than on the right). These are: (1) the genito-femoral running down near the aorta; (2) the ilio-inguinal passing down beneath the left ureter; (3) the ilio-hypogastric passing down and out beneath the left kidney; (4) the lateral cutaneous which accompanies the ilio-lumbal vessels. These nerves, illustrated by Greene (1955, fig. 176) are easily found just beneath the peritoneum in young animals. The nerve trunks were traced centrally to near the spine and distally to where they began to branch in the muscles; trunks and branches were excised. When chronic denervation was intended, rats of 70–80 g were used. After 10–14 d body weight was usually in the preferred range for cremaster observation (100–120 g).

*Preparation of the cremaster for histological examination.* Immediately after killing the animal with an overdose of pentobarbitone, the chest was opened and a catheter tied into the thoracic aorta, taking care to avoid entry of air into the vessel. The vessels were washed free from blood and fixed in the dilated state by perfusing under pressure (200 mm Hg) Ringer's solution and then formalin (Richardson, 1960) During the perfusion, fluid escapes from the vessels to distend the abdominal cavity and its extensions into the cremaster sacs. Formalin perfusion was continued for a half hour when thiocholine staining was intended; otherwise for 1–2 h. Then, the scrotum was removed, the cremasters separated up to their origin from the abdominal muscles and there cut off. The testis and epididymus were squeezed and pulled out from the sac, tearing the fine mesentery attaching them to the postero-medial wall. Sometimes the neck of the fixed sac is too narrow to allow the testis to pass without tearing the muscle; the neck is cut down one side to allow passage. Cremasters not wanted for immediate staining were stored in formalin. If required, the carcass of the rat was also stored in formalin.

Whatever the method of staining to be applied, at some stage or other, the cremaster sac was opened longitudinally, usually along the posterior fibrous zone. The fixed cremaster, unlike the unfixed (Majno, Palade & Schoefl, 1961), cannot be pinned flat without cutting off the pointed tip and making several radial cuts in the remainder. For most purposes it sufficed to cut the muscle into convenient portions. Before mounting, the subcutaneous tissue was stripped off, leaving the underlying muscle intact. The serosa may also be stripped but with difficulty and usually some tearing of the muscle. Towards the neck of the sac, the two muscle layers are easily separable so that the outer may be dissected off to provide a clearer view of the underlying vessels and nerves.

*Nerve staining.* Three methods were used.

(1) Gomori's (1952) modification of the thiocholine method provides a *general view of the nerve supply* (both myelinated and unmyelinated fibres) to the muscle and its vessels. The muscle, excised after a half hour formalin fixation, was washed in

running water for a half hour and then incubated for about 20 h at 37 °C with butrylthiocholine iodide in Gomori's solution. It was then processed as described, opened, spread, cleaned and mounted as a whole or in pieces in glycerine jelly.

(2) *Myelinated nerves* were stained with Sudan black B (Stillwell, 1957). Prior treatment with cobalt is unnecessary. About 15 min staining at room temperature is adequate. Preparations were mounted in glycerine jelly.

To trace the origin of any intact fibres persisting after nerve section and degeneration, the relevant portion of the carcass was treated as follows. The thorax tail, hind limbs, viscera and fat pads were removed. On the normal side the body wall and half the pelvis were cut away. The remaining portion of the carcass was washed in running water for an hour or two, treated with 50 and 70 % alcohol, about 1 h in each, and then immersed in alcoholic Sudan black for 1–2 h. After passing through the alcohols and water, the tissue was partially cleared by soaking in 50 % glycerine for about 20 h. The body wall was then pinned out in 50 % glycerine and examined under a magnification of 25 diam. with strong incident illumination. Beginning at the cut edge of the origin of cremaster, the peritoneum and underlying muscle were removed, clearly revealing the nerve strands. These were then related to those seen in the cut upper edge of the cremasteric sac and finally traced by further dissection to their origin from the spinal nerves. Tissue stained with Sudan black may later be impregnated with silver.

(3) *Axons* were impregnated with silver (Richardson's (1960) method). The muscle fibres are too darkly stained to allow vessels and nerves to be clearly seen in whole mounts except in the sparse and narrow interstices between the muscle bundles. To overcome this, before impregnation, the superficial connective tissue and as much as possible of the outer muscle layer were removed. The portion of the posterior wall containing the main blood vessels was excised and the remaining muscle cut into portions about 1 cm square. These pieces were impregnated separately except that when normal and denervated muscles were to be compared, portions of each were treated together. After impregnation, from the portion containing the main blood vessels the serosa and remaining muscle fibres were removed, leaving the vessels isolated. In the other portions, the muscle fibres were teased apart as far as possible after removing the serosa. The teased portions and the isolated vessels were then cleared in xylol and mounted in depex.

## RESULTS

*The cremaster muscle.* A knowledge of the anatomy is required to allow distinction between right and left excised muscle and orientation of an opened and spread sac.

Figure 1 shows a sac fixed in the distended state. The pointed tip is directed dorsally and a little mesially. This pointed tip is clearly seen during life when the testis is pushed down with the finger before inserting the stitch through the scrotum and the hind pole of the testis. Dorsally, and from tip to base, runs a narrow fibrous zone free or almost free from muscle fibres and from which arises the thin mesentery for the testis and epididymus. Muscle fibres are attached along each side of the fibrous zone. The muscle bundles are arranged in two layers which are extensions of the obliquus internus and transversus abdominis muscles. Over the basal portion

of the sac the two layers are distinct and easily separable; towards the tip the layers are less distinct and separation is difficult or impossible. The bundles of the superficial layer are for the most part circular or transverse in direction and those of the deep layer mainly longitudinal. But on the medial aspect the direction of the fibres is reversed, the superficial being longitudinal and the deep circular. The thickest part of the sac is a band of longitudinal fibres which lies laterally to the thin zone on the dorsal aspect. The part of the sac usually observed during life is on

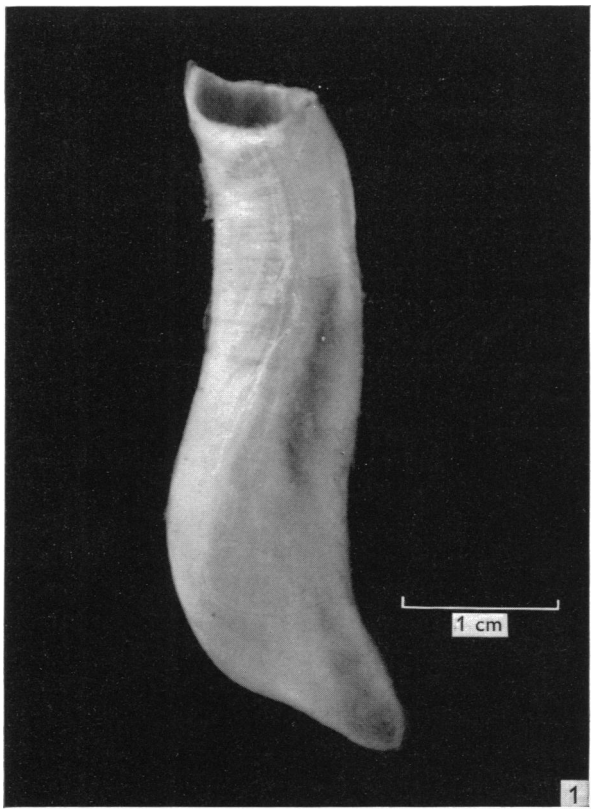


Fig. 1. Rat cremaster muscle, R, lying on side. Ventral aspect to left.

the curved belly on the ventral aspect of the sac. The main blood vessels run down the posterior wall and pass branching outwards between the two muscle layers. They arise from the external spermatic vessels. A small artery enters the tip of the cremaster which, according to Schoefl (1963), is derived from the epididymal branch of the internal spermatic artery.

*The nerve supply.* Whole mounts stained with butyrylthiocholine provide a general view of the nerves, both myelinated and unmyelinated. As Fig. 2 shows, the muscle fibres are but lightly coloured; faint parallel and criss-cross lines indicate the two layers. Against this background, the nerves supplying the muscle and its end plates are conspicuous as are also the nerve strands accompanying the blood

vessels. Muscle spindles are absent. Addition of Sudan black shows that the nerves going to the end plates are myelinated while those accompanying the vessels have no sheath stainable by this method.

All the nerves enter the cremaster by the neck of the sac in a series of 10 to 15 parallel strands which pass down the sac towards the tip, branching and intercommunicating. The strands are distributed mainly to the muscle, only a few go to the blood vessels.

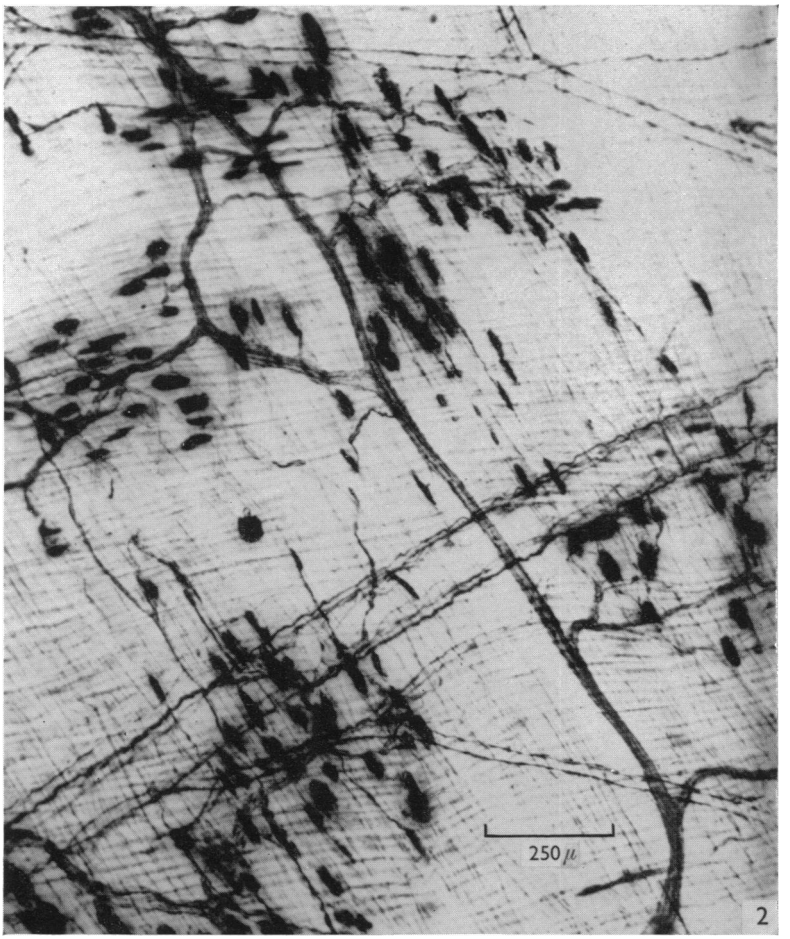


Fig. 2. Rat cremaster, portion of whole mount. Butyrylthiocholine preparation.

Local concentrations of the perivascular nerves that might indicate the presence of arteriovenous anastomoses (Grant & Thompson, 1963) are wanting.

Though the thiocholine method serves well for demonstrating the normal nerves, it is not helpful after nerve section. So much staining persists 10–14 d later that distinction of the degenerate from normal is impracticable. For this purpose reliance has been placed on silver impregnation and Sudan black.

Figure 3, from a silver impregnated preparation, shows that the cremaster vessels are supplied with a perivascular plexus of the usual type, and presumably sympathetic in origin.

Five rats were killed 7–19 d after removal of the abdominal sympathetic nerves; since the chains are conjoined in the rat, unilateral removal is not feasible. The plexus in the operated animals seemed to be but little reduced when compared with that in normal rats of the same body weight. This is not surprising since it is known that


Fig. 3. Nerve plexus on isolated arteriole. Normal cremaster. Richardson's silver impregnation.

many intermediate ganglia are present in the rat's lumbar region (Wrete, 1941). But, when all the myelinated nerves to the cremaster were cut and degenerate, then no, or almost no, perivascular plexus could be demonstrated.

According to Greene (1955), the cremaster is supplied by the genito-femoral nerve. But section of this nerve, though causing most of the myelinated fibres to degenerate, left a variable number intact and did not much reduce the perivascular fibres (4 rats, killed 14–33 d after operation). Dissection of the normal body wall

stained with Sudan black showed that strands from the ilio-inguinal and ilio-hypogastric nerves join those of the genito-femoral. That these two nerves may contribute to the cremaster supply was shown by cutting each of them separately. Thus, in two rats, the ilio-inguinal and in two others the ilio-hypogastric nerves was cut. In one of each pair, a few degenerate strands were found in the cremaster nerves.


Fig. 4. Degenerate branching nerve strand, containing two intact and branching myelinated fibres, 15 days after nerve section. Sudan black.

In a series of fourteen rats with all three nerves cut and killed 10–15 d after operation, a variable number of intact fibres was found in the cremasters of seven. Dissection of the body wall stained with Sudan black in several which showed persisting intact strands revealed two further sources of supply to the cremaster. In one rat, four intact fibres in the neck of the cremaster were traced upwards to join a branch of the lateral cutaneous nerve. Figure 4 shows one of the degenerate strands in this animal containing two intact fibres. The distinction between the normal and degenerate is readily made. In another rat, among the degenerate strands entering the cremaster were two composed mainly of intact fibres. Traced upwards, these joined with others within the genito-femoral trunk. The distal cut end of this trunk was found fixed to the underlying muscle. Near the upper end

of the cut trunk, the strand of intact fibres left the degenerate nerve and entered the caudal muscles about the level of the bifurcation of the common iliac artery. The origin of this intact strand was not determined, but it presumably arose from one of the upper sacral nerves. A fine communicating branch, containing some myelinated fibres, joined this intact strand to the abdominal sympathetic chain. These myelinated fibres did not pass distally but turned centrally along the sacral strand. This example emphasizes the need to follow the nerves as far distally as possible before cutting and excising.

Finally, in a series of seventeen rats, the genito-femoral, ilio-inguinal, ilio-hypogastric and lateral cutaneous nerves were cut and excised. The animals were killed 7–13 d later. In fifteen, no intact myelinated fibres were found, but one intact fibre was found in each of the remaining two. The source of these was not determined. Silver impregnation revealed a few strands of perivascular plexus in these two and in one other in which no intact myelinated fibres were discovered. It seems clear, therefore, that section of these four nerves denervates the cremaster and its blood vessels completely or virtually so. These animals were used for testing the vascular effects of denervation.

*Vascular effects of denervation.* Preliminary observations on two normal animals showed that when the two cremasters were exposed simultaneously, they behaved alike in the subsidence of initial dilatation (due to the disturbance of preparation) and responded in the same way to vasoactive substances applied topically and intravenously (adrenaline, histamine and acetylcholine).

Both cremasters were so exposed in five animals, 30 min to 1 h after cutting the four nerves on the left side. In all five succinylcholine chloride (100 mg/100 ml) was applied to quieten the muscle. This, as usual, caused a transient increase in the flickering movements on both sides before the muscles became quiet and relaxed. In one animal, the initial dilatation persisted on both sides but in the other four that on the normal side subsided as usual. In these four the dilatation on the operated side decreased to some extent but the vessels did not narrow to the same degree as on the normal side. The dilatation seemed to involve the arteries more than the minute vessels. Histamine and adrenaline applied topically provoked dilatation and constriction on both sides. The degree of change on the two sides could hardly be compared because of the unilateral dilatation, but there was no obvious difference in the two reactions.

In a series of eleven rats, both cremasters were exposed simultaneously 8–18 d after left-sided denervation. Again both sides displayed vaso-dilatation and flickering movements. Irrigation with succinylcholine provoked a more vigorous response from the denervated than from the normal cremaster; the muscle contracted strongly and its vessels were squeezed into corkscrew-like folds. This stimulation soon passed and both muscles became quiet and relaxed. Dilatation persisted on both sides in three animals and these were discarded. In the remaining eight, it subsided equally on the two sides. Vasoactive substances were tested on these.

Adrenaline, applied topically to seven, gave the same degree of constriction on both sides in five, the minimum effective concentration (M.E.C.) being 0·1–0·01 ng/ml; in one the M.E.C. appeared to be ten times greater on the left denervated side but in the other the reverse was the case. Given intravenously to three, adrenaline

provoked equal dilatation followed by constriction on the two sides, the minimum effective dose (M.E.D.) being 100 ng for each side.

Acetylcholine applied topically in concentrations decreasing from 1 $\mu$g/ml did not stimulate the denervated muscle to contract but provoked the usual arterial dilatation on both sides. In two, the responses were equal, the M.E.C. being 0·1 ng/ml; in the third, the M.E.C. was estimated as 1·0 ng/ml for the normal and doubtfully as 0·1 for the denervated.

Equal responses on the two sides were obtained from histamine (three rats) and 5-hydroxytryptamine (5-HT) (two rats) applied topically.

The sensitivity was further tested in another series of eleven rats in which only the denervated cremaster was exposed 8–15 d after operation. The M.E.C. and M.E.D. were estimated and later compared either with those previously determined (Grant, 1964) or where this had not been done, newly determined. The results are summarized in Table 1.

Table 1. *Response of normal and denervated cremasters to vasoactive substances*

| | Topical application. Minimum effective concentration (ng/ml) | | Intravenous application. Minimum effective dose (ng) | |
|---|---|---|---|---|
| | Denervated | Normal | Denervated | Normal |
| Adrenaline | 0·1 (3)<br>0·01 (1) | 0·1–0·01* | 100 (2)<br>10 (2) | 100–10* |
| Noradrenaline | 0·01 (1)<br>0·001 (2) | 0·01–0·001* | 100 (1)<br>10 (2)<br>1 (1) | 100 (1)<br>10 (1)<br>1 (2) |
| Acetylcholine | 1·0 (3)<br>0·1 (2) | 0·1–0·01* | 100 (3)<br>10 (2) | 100 (2)<br>10 (2)<br>1 (1) |
| Histamine | 1·0 (1)<br>0·1 (3) | 0·1–0·01* | 1000 (1)<br>10 (1)<br>1 (2) | 1000 (1)<br>10 (1)<br>1 (2) |
| 5-Hydroxytryptamine | 0·1 (3)<br>0·001 (2) | 0·001* | 1000 (1)<br>100 (3) | 1000 (1)<br>100 (2) |

Numbers in parentheses = number of rats.
* = previously reported values (Grant, 1964).

It seems fair to conclude from these observations that, though nerve section is followed by a temporary vasodilatation and that though the denervated muscle becomes hypersensitive to succinylcholine, an acetylcholine-like substance, yet the denervated vessels show no indication of any increased sensitivity to various vasoactive substances applied topically or intravenously. If anything, in the second series of eleven rats, the response to topical acetylcholine is on the whole less than normal.

As before (Grant, 1964) the M.E.C. and M.E.D. were determined by applying successive tenfold dilutions. Attempts to make a closer estimation by using five- and twofold dilutions gave equivocal results, because of the difficulty of subjectively distinguishing very fine differences in response.

*Nerve stimulation.* A number of observations were made to see if constriction or dilatation could be provoked by nerve stimulation, direct and reflex.

In three rats, through the operative approach for a sympathectomy, shielded electrodes of silver wire were applied to the sympathetic chain at the level of the left kidney. Faradic stimulation was applied with peak voltages ranging up to 50 V at the rate of 50/s for 2–30 s. In all three an inconstant slight vasoconstriction was obtained. In one rat slight dilatation followed the constriction or occurred alone, again inconstantly. Because of these inconclusive results and of the difficulty of satisfactorily applying the electrodes to the delicate sympathetic chain, these experiments were not pursued. The same faradic stimulation was applied in two rats to the moistened skin of the foot and ear and also to the exposed femoral nerve. Again inconstant constrictor and dilator responses were obtained. In two other rats the carotid arteries were exposed; occlusion of one or both failed to provoke any change in the cremaster vessels.

## DISCUSSION

For comparison with these results, little seems to have been recorded about the vascular effects of denervation in skeletal muscle. What there is is mainly in terms of blood flow and in none of the studies are the effects of nerve section on the perivascular plexus determined. It is to be remembered, however, that hypersensitivity may develop, although a considerable amount of the perivascular plexus persists after nerve section (rabbit ear, Grant & Thompson, 1963).

On only one point is agreement general, namely, that denervation, sympathetic or total, is immediately followed by a vasodilatation which subsides within a few days (Dale & Richards (1918–19) and Hilton (1959) for the cat; Griffin, Green, Youmans & Johnson (1954) for the dog and Grant & Pearson (1937–38) for man; see Shepherd (1963) for other references to man).

While there is thus general agreement about what is commonly called the regain of vascular tone after denervation, the findings about the response of the chronically denervated vessels to vasoactive substances are conflicting. According to Dale & Richards (1918–19) the dilator effect of small doses of histamine, adrenaline and acetylcholine is habitually much larger in the cat's denervated limb than in the normal (volume records). Hilton (1959) also in the cat's limb (muscle blood flow) 8–15 d after nerve section finds the vascular responses to dilator substances such as acetylcholine and histamine are 'enormously enhanced'. On the other hand, Winbury (1959), measuring total limb flow in the cat, records that acetylcholine and bradykinin produce the same effect in normal and denervated limbs, though the dilator effect of serotonin (5-HT) is blocked by the denervation. Grant & Pearson (1937–38) in one patient suffering from thromboangiitis, found a greater dilator response of the forearm to adrenaline than normal when tone had been regained after sympathectomy. So also Stein, Harpuder & Byer (1949) and Duff (1953) for the sympathectomized human leg and forearm provide evidence for increased dilator and constrictor effects from adrenaline. According to Griffin *et al.* (1953), however, the responses of the dog's denervated hind limb to adrenaline and noradrenaline are unchanged; their normal response to adrenaline, like that of

noradrenaline, is constrictor. Cobbold & Vass (1953) see no indication of increased reaction to noradrenaline in the cat's hind limb after sympathectomy.

There is no obvious explanation for these discrepancies and it is clear that further study is required for their resolution. It is believed that skeletal muscle is supplied with both constrictor and dilator nerves (Green & Kepchar, 1949). The observations on the cremaster, however, do no more than suggest that its vessels, like those of other skeletal muscles, are supplied with both constrictor and dilator nerves. The temporary dilatation after nerve section also suggests the removal of constrictor control. From what is known about denervation in general (Cannon & Rosenblueth, 1949) it might be expected that degeneration of these nerves would lead to an enhanced sensitivity to both constrictor and dilator substances. But it is also believed that since intrinsic tone of skeletal muscle vessels is high, nervous control is correspondingly weak (Barcroft, 1963). It may be, therefore, that the degree of sensitivity after denervation is also weak, and the results of Duff (1953) and Stein *et al.* (1949) suggest that this is so in man. If this is so, then the method used may not be sufficiently delicate to detect small changes of sensivity. But it is clear that in the cremaster there is no gross change corresponding to Hilton's (1959) 'enormous' enhancement or even to that in the rabbit's denervated ear where the sensitivity is of the order of 100–1000 times greater than normal (Armin, Grant, Thompson & Tickner, 1953).

SUMMARY

1. The nerve supply to the rat's cremaster muscle was studied in relation to effecting denervation of the blood vessels.

2. Abdominal sympathectomy does not, but section of the spinal nerves to the muscle does, lead to disappearance of the perivascular plexus.

3. As judged by direct observation of the vessels, their sensitivity to vasoactive substances is not altered by denervation.

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