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SOME OBSERVATIONS ON THE CONTRACTILE TISSUE OF THE MAMMARY GLANDS

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Recent histological studies (Richardson, 1949; Linzell, 1952; Silver, 1954) have provided strong circumstantial evidence for the view that the mammary myoepithelium is a contractile tissue, which produces milk ejection in response to oxytocin released from the posterior pituitary gland during suckling. However, direct evidence of contractility is lacking.

A number of other substances have been reported as causing partial or complete milk ejection in the isolated perfused cow's udder (Petersen, 1942; Peeters, Sierens & Silver, 1952), but it cannot be decided with certainty whether they are acting upon the myoepithelial cells around the alveoli or upon the smooth muscle of the teat and cistern, which has been shown to contract in response to many drugs as well (Peeters, 1948).

Direct microscopical examination of the living mammary gland has been undertaken to determine the site of action of the substances producing the expulsion of milk and to study the response of the myoepithelial cells. Preliminary results have been previously reported to the Physiological Society (Linzell, 1954).

METHODS

Lactating mice were used as the main experimental animals but comparisons were made with the rat, rabbit and guinea-pig. Rats and mice were anaesthetized with 10% (w/v) urethane by intraperitoneal injection, 150 and 170 mg/100 g respectively; some mice needed more, up to 220 mg/100 g. A solution of 1% (w/v) chloralose and 10% (w/v) urethane (75 and 750 mg/kg) was used for rabbits and guinea-pigs, given by intravenous and intraperitoneal injection respectively.

The mammary glands were exposed and observed with a stereoscopic dissecting microscope ($\times 20$ to $\times 50$). In the rat and mouse it is often convenient to reflect the skin and adherent glands, which can be laid on a flat surface without interfering with the blood and nerve supply. The dorsal (abdominal) side of the mammary tissue is then examined. The exposed tissue was kept moist with physiological saline solutions (usually that of Krebs & Henseleit, 1932) and covered with a sheet of thin polyethylene.

Incident illumination was used from a shielded Pointolite lamp focused on the tissue through a ½ in. Perspex rod, which acted as a heat filter. Photographs were made with a low-power

monocular microscope and orthochromatic plates either with the same illumination (exposure time ¼–1 sec) or latterly with a 100 J electronic flash apparatus synchronized with the camera shutter (flash duration 2 msec).

Drugs were made up in 0·9% (w/v) NaCl solution. For topical application the solutions were delivered, in volumes of 0·0002–0·1 ml., from a micrometer syringe (Burroughs Wellcome and Co.), as drops at the tip of a 20-gauge needle and gently placed on the tissue without touching it with the needle. For the determination of threshhold the volume was kept constant at 0·0002 ml. Doses of the pituitary hormones are expressed in milliunits (mU).

Electrical stimulation was carried out with small bipolar or unipolar electrodes supplied from an a.c. source at 50 c/s or a square-wave stimulator.

In some experiments the milk-ejection responses of the whole gland to intravenously administered drugs were observed simultaneously with the local response. In the guinea-pig and rabbit the teat was cannulated and milk outflow recorded on a kymograph. In the mouse the flow of milk into the main teat duct was observed microscopically.

RESULTS

Appearance of the living mammary gland

In the rat, mouse and rabbit the lactating mammary glands have a thin covering of connective tissue which is almost completely transparent. There are a variable number of individual fat cells, which do not seriously obstruct vision, but make photography of the mammary tissue difficult. In the well nourished guinea-pig there is a much thicker layer of fat lobules which have to be parted to reveal the mammary tissue. When they contain milk the surface alveoli appear as gleaming white structures by incident illumination in contrast to the pink surrounding tissue, each lobe resembling a somewhat compressed bunch of irregular grapes (Pls. 1 and 2). The empty alveoli are also pink and are therefore more difficult to see (e.g. Pl. 1, fig. 4, and Pl. 2, fig. 16). To ensure that the glands contained some milk, the litters were removed 1 to 14 hr before using the animals. The rate of filling varies with the stage of lactation, the size of the litter and whether the animal is simultaneously pregnant. In the mouse all the glands may be completely emptied by vigorous sucking so that no alveoli containing any milk can be found. After a period of 4–6 hr the alveoli of all animals contain some milk and this was the usual interval used.

Examination of some animals revealed that not all the alveoli were terminal structures in the gland tree, but that the outlets of many joined other alveoli rather than ducts. This was most easily seen in the glands of animals with small litters, or in those starting to involute. Mosimann (1949) has described the same condition in reconstructions from serial sections of the mammary gland of the cow and refers to this arrangement as that of a branched alveolar gland. All the alveoli, whether terminal or not, are lined by secretory cells and cannot ordinarily be distinguished from each other.

The large ducts, apart from a very few running near the gland surface (Pl. 2, figs. 11–16), are not usually visible in the fully active gland unless milk

ejection has just taken place. When the gland has involuted to the stage at which the alveoli no longer contain milk, the ducts are more easily seen (see later). Capillaries can be seen running over the surface of the alveoli.

The living myoepithelial cells cannot be distinguished by normal, ultraviolet or polarized light and so far attempts to stain them vitally or supra-vitally have failed. Phase-contrast microscopy of simple squash preparations has not been helpful because it renders all cells, fibres and the abundant fat globules more easily visible and the myoepithelium is obscured.

Evidence for the contractility of alveolar myoepithelial cells

The alveoli themselves have been seen to contract in response to the same physiological and pharmacological stimuli which produce contraction of smooth muscle. The contained milk was partly or completely squeezed out into the ducts and then more gradually ran back again (Pls. 1, 2). The time course of the response was also similar to that of smooth muscle; 2–5 sec for contraction and 0·5–5 min for relaxation after a latent period of 2–20 sec. If the glands were almost empty the milk did not return to the alveoli, which remained contracted. If the glands were very greatly distended (e.g. in the mouse 12 hr after last suckling) some alveoli did not contract, presumably because the pressure of milk was too great (Pl. 2, figs. 20–24). It was possible to obtain another contractile response as soon as relaxation had taken place. However, with too frequent stimulation the preparation became insensitive. With most animals tests could be repeated every 5 min for 2–3 hr on the same area of mammary tissue; a few were still active after 3–5 hr (Pl. 2, figs. 17–24).

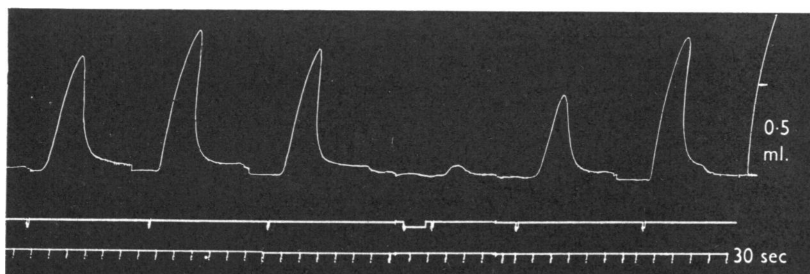
The structures taking part in this contractile process are the lining alveolar cells, the well-developed network of myoepithelial cells embracing them, and the adjacent intralobular scanty connective tissue and capillaries. Smooth muscle is not present in the intralobular connective tissue (Richardson, 1949; Linzell, 1952). There is no evidence that connective tissue, devoid of smooth muscle, or capillaries in other situations are capable of exerting a powerful sudden compression or that the acinar cells themselves could produce it. However, the myoepithelial cells are ideally arranged and situated for this purpose, and it was therefore concluded that they are the contractile elements as suggested by the early German histologists on purely morphological grounds (see Linzell, 1952).

Stimuli producing alveolar contraction

Electrical stimulation

Stimulation with bipolar electrodes produced contraction of four to five alveoli near each electrode (Pl. 1, figs. 1, 2), but a unipolar electrode produced a more widespread response of 1–3 mm^2 (Pl. 1, figs. 3, 4; Pl. 2, fig. 15). The response was unaffected by D-tubocurarine (1:1000). Mechanical stimuli, such

as brushing with cotton-wool, produced a widespread alveolar contraction of surface alveoli in a few animals. Stimulation of the cutaneous nerves, supplying the glands, with a wide range of voltage, frequency and pulse duration, had no effect. Moreover, stimuli, which were known to excite the adrenergic sympathetic vasoconstrictor fibres in other species (Linzell, 1950), prevented the action of intravenous oxytocin or vasopressin if applied shortly before the injection (Text-fig. 1), but did not antagonize their topical action.



Text-fig. 1. Milk-ejection responses to repeated doses of 2 mU pure oxytocin (du Vigneaud's) given intravenously at 5 min intervals to an anaesthetized guinea-pig. Volume records of the milk outflow into a vertical cannula (3 mm internal diameter) in the main teat duct. Immediately before the fourth injection the external spermatic nerve was stimulated (5 V; 1 msec; 50/sec). The drum was stopped between responses.

Parasympathomimetic drugs

Acetylcholine chloride (Pl. 2, figs. 11, 12, 19 and 22) applied to the surface lobules produced complete alveolar contraction and capillary dilatation in small amounts (threshold 0·00001– 0·01 μg). Pilocarpine chloride (0·002–1·0 μg) (Pl. 2, figs. 11, 13, 19 and 20) and the chlorides of other choline esters were also effective. Comparative thresholds in one experiment were acetylcholine 0·0001 μg, carbamylcholine 0·0002 μg, acetyl-β-methylcholine 0·002 μg and benzoylcholine 0·02 μg. In some experiments the threshold dose of acetylcholine (ACh) was lowered by previous eserinization of the whole animal or the topical applications of eserine. Atropine always abolished ACh action.

Examination of mouse mammary glands by the histochemical method of Koelle (1950) for cholinesterases revealed small amounts of pseudo-cholinesterase in the connective tissue cells and the cytoplasm of fat cells, but no true cholinesterase.

Sympathomimetic drugs

Adrenaline hydrochloride and L-noradrenaline bitartrate (Pl. 1, figs. 5, 6) produced capillary and arteriolar constriction only, even in large amounts (1 μg). However, they did not prevent other active substances (e.g. acetyl-

choline, oxytocin), applied topically at the height of the vasoconstriction from producing their usual alveolar contraction (Pl. 1, figs. 7, 8). Moreover, when adrenaline and oxytocin were made up in the same solution, vasoconstriction and alveolar contraction occurred simultaneously upon topical application. In two experiments the threshold to oxytocin was unaltered by the addition of adrenaline (0·0005 and 0·001 μg) to the solutions, whereas in one the intravenous threshold to oxytocin was raised from 0·25 to 0·7 mU by the simultaneous administration of adrenaline (1 μg/1 mU of oxytocin). In other experiments the intravenous threshold did not alter in this way, and was the same when determined by observing glands on opposite sides of the same animal.

Posterior pituitary hormones

In addition to commercial posterior pituitary extracts and the partially separated hormones (Pitocin and Pitressin, Parke Davis), the purest samples of vasopressin and oxytocin of du Vigneaud produced alveolar contraction in extremely small amounts (Pl. 1, fig. 8; Pl. 2, figs. 16, 23 and 24). The ratios of

TABLE 1. Threshold doses of pure oxytocin and vasopressin of du Vigneaud (mU)

	Intravenous			Topical		
Species	Oxytocin	Vasopressin	V/O	Oxytocin	Vasopressin	V/O
Mouse	—	—	—	1×10^{-8}	1×10^{-8}	1
	—	—	—	2×10^{-7}	1×10^{-5}	50
	0·3	3	10	2×10^{-4}	2×10^{-3}	10
	—	—	—	2×10^{-5}	1×10^{-4}	5
	0·15	2	13·3	—	—	—
	0·25	4	16	5×10^{-5}	1×10^{-4}	2
	0·25	3	12	2×10^{-6}	2×10^{-5}	10
	0·2	3·7	18·5	2×10^{-5}	1×10^{-4}	5
	0·1	1·25	12·5	—	—	—
	0·2	2·0	10	2×10^{-6}	2×10^{-5}	10
	0·15	2·0	13·3	2×10^{-6}	2×10^{-4}	100
	0·4	6·0	15	2×10^{-6}	2×10^{-5}	10
Mean	0·22	3·0	13·4	$3{\cdot}0\times10^{-5}$	$2{\cdot}6\times10^{-4}$	20
Calculated from means		—	13·6	—	—	8·7
Guinea-pig	2	15	7·5	2×10^{-11}	1×10^{-7}	5000
	1·5	15	10	—	—	—
	2	25	12·5	—	—	—
	1	12·5	12·5	5×10^{-6}	5×10^{-6}	1
	1	15	15	2×10^{-6}	2×10^{-5}	10
Mean	1·5	16·5	11·5	—	—	—
Calculated from means		—	11·0	—	—	—

activity of vasopression and oxytocin (V/O) were determined in a few experiments by comparison of thresholds (Table 1), which are given as the smallest amount to produce a detectable contraction. For complete alveolar contraction 100 times the threshold dose was usually required. The stock solutions kindly provided by Professor du Vigneaud were prepared by him from arginine-vasopressin assaying at 400 U/mg and oxytocin at 450–500 U/mg. As will be seen

from Table 1, the variation in sensitivity to the topically applied hormones was very great as was also the ratio of activities by this method. However, the mean values for the latter agree fairly well with those obtained by the intravenous route and together suggest that oxytocin was 12 times as active as vasopressin. The sensitivity to pure vasopressin and oxytocin was not significantly different from that to the commercial Pitressin and Pitocin and in three experiments in which the thresholds to Pitocin and oxytocin were compared in the same animal they were found to be the same.

Neither atropine (Pl. 2, fig. 24), dibenamine (in doses sufficient to inhibit the actions of acetylcholine and adrenaline respectively), D-tubocurarine (1:1000) nor procaine prevented the action of oxytocin.

Other drugs

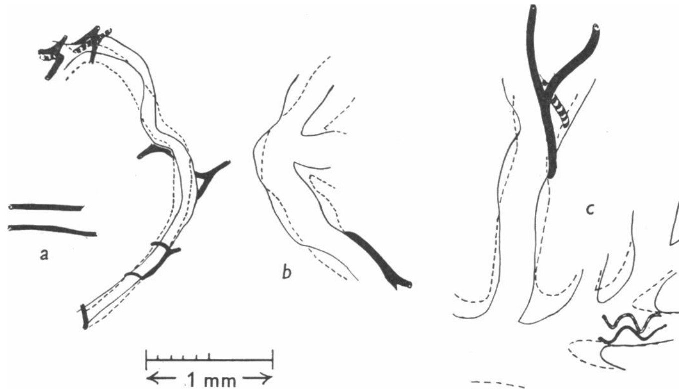
Histamine acid phosphate 0·05–1·0 μg (Pl. 2, figs. 11, 14, 17 and 18), barium chloride 1–50 μg (Pl. 2, figs. 19, 21), and 5-hydroxytryptamine creatinine sulphate 1 μg (Pl. 2, figs. 9, 10) all produced alveolar contraction. Marked tachyphylaxis was seen with 5-hydroxytryptamine; sometimes only one response was obtainable from a given area.

The duct myoepithelium

The myoepithelial cells of the ducts take the form of longitudinally arranged aciculate cells, which would tend to shorten and widen the ducts if they were contractile. On the few occasions when they could be observed in the fully active gland the ducts did become wider during generalized alveolar contraction of a whole lobule (Pl. 2, figs. 11–16; and Linzell, 1952, figs. 14 and 15). However this might be entirely due to passive distension by milk squeezed from the alveoli.

In the mouse it was found that if the litter was reduced to 2, some glands were not sucked and started to involute. After a week the alveoli were greatly reduced in size and contained no milk; they have been independently described by Cole (1933) as collapsed and degenerating at this stage of involution. However, some large and medium-sized ducts remained and were easily visible since they still contained some milk. In this state they were photographed in three experiments (with a micrometer eyepiece in the optical set-up) before and just after the topical application of pure oxytocin (0·002 mU). After photographic enlargement to a final magnification of 50–100, measurements of the duct width were made by two observers every 100 μ along the length of the area under test. Tracings were also made and carefully superimposed using large blood vessels in the field as landmarks (Text-fig. 2). In each case there was a significant widening of the ducts, and in two an obvious straightening and shortening as well. The duct in Text-fig. 2*a* had a mean width of $88 \pm 23\,\mu$ (S.D.) before and $104 \pm 23\,\mu$ after oxytocin ($P = 0{\cdot}01$); its

length measured between the vessels crossing it at the top and bottom decreased 8 %. The duct in Text-fig. 2*b* increased in mean width from $234 \pm 25\mu$ to $286 \pm 54\mu$ ($P = 0{\cdot}001$). In Text-fig. 2*c* the duct running into the main duct increased from $306 \pm 38\mu$ to $341 \pm 41\mu$ ($P = 0{\cdot}02$); its length, measured from the bifurcation of the large blood vessel to the edge of the main duct, decreased by 10 %. The shortening of another tributary of the main duct is also shown by the decrease in 'wavelength' of the undulating blood vessel on its surface.



Text-fig. 2. Superimposed tracings of ducts made from photographs of three partially involuted mouse mammary glands. Solid lines before and broken lines after topical application of pure oxytocin 0·002 mU. Some of the blood vessels used as landmarks are included. Those that moved are also shown interrupted in their new positions (see also text).

DISCUSSION

The evidence presented for the contractility of the mammary myoepithelium, together with the fact that it responds to such extremely small amounts of oxytocin, confirms the now generally accepted view that this tissue is the final effector structure concerned in the ejection reflex. All the stimuli producing alveolar contraction are also those that cause smooth muscle to contract, so that the milk ejection seen in the perfused cow's udder in response to pharmacological agents (Petersen, 1942; Peeters *et al.* 1952) may be due to stimulation of the mammary myoepithelium and/or the smooth muscle of the cistern. The response to 5-hydroxytryptamine is of interest because it probably accounts for the milk-ejecting activity of shed blood (Peeters *et al.* 1952) in which this substance is known to develop after the rupture of blood platelets (see Page, 1954).

Dempsey, Bunting & Wislocki (1947) have suggested that the mammary myoepithelium is concerned with the transport of metabolites. This was because they found alkaline phosphatase in the resting gland in the outer cell layer of the ducts, but failed to find it in the lactating gland. Richardson (1949) has emphasized the need to distinguish carefully between the two types

of fully differentiated myoepithelial cells of the active gland on the one hand, and the immature outer duct epithelium on the other. The histologists of the last century believed that the former were derived from the latter, and presumably for this reason Dempsey *et al.* (1947) refer to the outer cells of the immature ducts as myoepithelium. To avoid confusion it would be better to restrict this term to the fully differentiated cells, which Silver (1954) has now shown in fact do contain alkaline phosphatase. If the mature myoepithelial cells are concerned with the transport of metabolites, this function would appear to be in addition to their role in the evacuation of milk from the gland.

Although Arnstein (1895) described nerve endings on some isolated cells of the mammary gland in the pregnant cat, when supravitally stained with methylene blue, more recent studies by silver techniques (Dempsey *et al.* 1947; Linzell, 1951, 1952) have failed to demonstrate innervation of either secretory or myoepithelial cells. Physiological evidence for the lack of specific secretory nerves to the mammary glands is abundant. The present observations show that not only does nerve stimulation fail to produce milk ejection from the whole gland (see also Peeters, Coussens & Sierens, 1949; Linzell, 1950), but also fails to cause contraction of the myoepithelial cells or any movement of milk from the alveoli. These experiments then provide no support whatsoever for the theory of Hammond (1936), that milk ejection is due to a vascular erection mediated by vasomotor nerves.

The examination of the living mammary gland appears to have settled a controversy. In the whole animal adrenaline or sympathetico-adrenal activity, antagonizes the action of endogenous or exogenous oxytocin (Ely & Petersen, 1941; Cross, 1953). It does not have this action if given just before or with oxytocin to a gland perfused at constant volume inflow (Linzell, 1951). The present observations confirm the inhibitory action of adrenaline by the intravenous route, and show that stimulation of the sympathetic nerves to the gland has a similar action. It is now clear that neither adrenaline nor noradrenaline inhibit the myoepithelium itself when active drugs are applied topically at the height of the vasoconstriction. Therefore the peripheral inhibitory action of sympathetic-adrenal activity on milk ejection in the whole animal is most likely to be due to an intense vasoconstriction preventing adequate access of oxytocin to the myoepithelium, because it is known that the mammary blood vessels are very sensitive to adrenaline and noradrenaline (Hebb & Linzell, 1951) and that stimulation of the sympathetic nerves can temporarily stop the mammary blood flow (Linzell, 1953, fig. 6). In the gland perfused at constant volume inflow the oxytocin reaches the gland in spite of vasoconstriction.

The action of highly purified vasopressin in producing alveolar contraction confirms the view of Popenoe, Pierce, du Vigneaud & van Dyke (1952) that this polypeptide has an inherent oxytocin-like activity of its own. The ratio

of activities of purified oxytocin and vasopressin (12:1) determined in this work was of the same order as that reported for the rabbit by Cross & van Dyke (1953), and for the sow by Whittlestone (1952). The great variations in sensitivity to the topically applied hormones is not surprising. With this unphysiological method of application, variations in the area of spread, rates of diffusion, thickness of connective tissue covering and amounts and location of destructive enzymes (Page, 1946) might all account for this phenomenon.

SUMMARY

1. By direct microscopical examination of the living mammary glands the alveoli have been shown to be capable of contraction so that the contained milk is forced out into the ducts.

2. The following stimuli produced alveolar contraction, when applied topically:

(*a*) The highly purified pituitary hormones vasopressin and oxytocin of du Vigneaud. Pure vasopressin had an activity of some 8% of that of pure oxytocin.

(*b*) Direct electrical and sometimes mechanical stimulation.

(*c*) Acetylcholine, carbamylcholine, benzoylcholine, acetyl-β-methylcholine, pilocarpine, histamine, barium chloride and 5-hydroxytryptamine.

3. Adrenaline, noradrenaline and sympathetic nerve stimulation had no action on the alveoli and did not prevent alveolar contraction in response to other stimuli. It is suggested that their peripheral-inhibiting action is due to vasoconstriction which normally prevents oxytocin from reaching the myoepithelial cells.

4. The ducts, around which the myoepithelium is arranged longitudinally, have been shown to widen and shorten in response to oxytocin at a stage of involution when the alveoli are degenerating and no longer contain milk.

I should like to express my sincere thanks to Prof. V. du Vigneaud for samples of his highly purified vasopressin and oxytocin; to Dr W. Feldberg, F.R.S., for 5-hydroxytryptamine; and to the Imperial Chemical Industries for pure polyethylene sheet. The cholinesterase staining was kindly carried out by Dr Catherine O. Hebb.

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EXPLANATION OF PLATES

Photographs of living mammary glands of lactating mice. P = panniculus carnosus muscle; D = duct; F = fat tissue. Magnification × 17·5, except in Figs. 1 and 2 (× 36) and 9 and 10 (× 26).

PLATE 1

Figs. 1 and 2. Bipolar electrical stimulation with one electrode near edge of mammary gland at *st*. Localized contraction of alveoli.

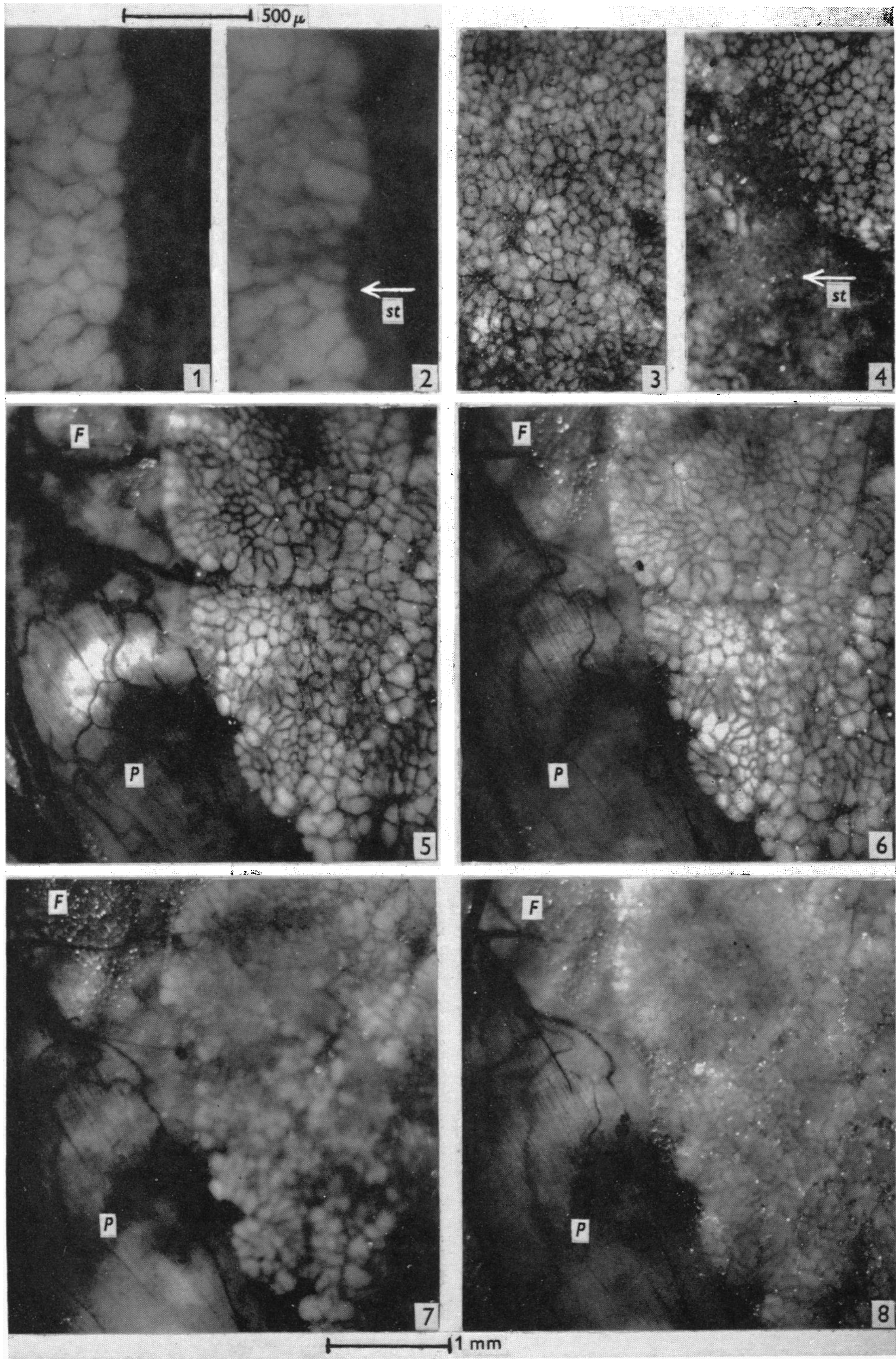
Figs. 3 and 4. Unipolar electrical stimulation of gland surface at *st*. Widespread alveolar contraction.

Fig. 5. Edge of gland, panniculus muscle and small blood vessels. 2.50 p.m.

Fig. 6. Same area just after topical application of L-noradrenaline bitartrate 0·0001 μg in 0·1 ml. Blanching of gland and constriction of small blood vessels. 2.55 p.m.

Fig. 7. Acetylcholine chloride 0·001 μg in 0·1 ml. applied at height of vasoconstriction. Patchy alveolar contraction in spite of it, throwing some parts out of focus. 2.56 p.m.

Fig. 8. Oxytocin 0·00001 mU in 0·1 ml. applied 1 min after same amount of noradrenaline. 3.28 p.m. Uniform alveolar contraction.



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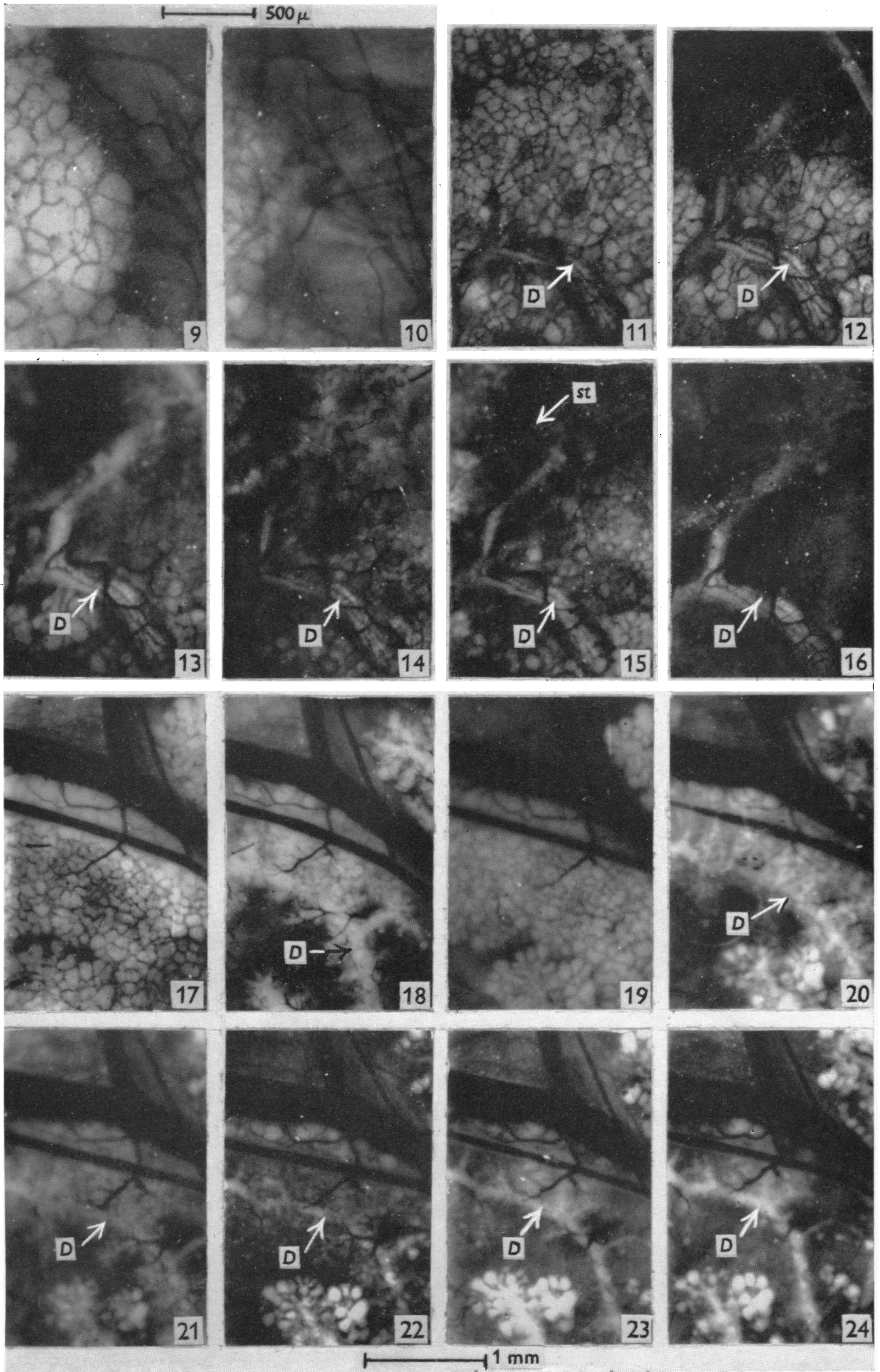
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PLATE 2

Fig. 9. Edge of distended lobe after saline control. No effect on alveoli or vessels.

Fig. 10. 5-Hydroxytryptamine creatinine sulphate 1·0 μg in 0·1 ml. Alveolar contraction.

Figs. 11–16. Repetitive responses from same area of gland. Between each response the alveoli relaxed to the condition shown in Fig. 11. Stimuli applied to top of field in 0·0005 ml. Fig. 11. Control. 3.30 p.m. Fig. 12. Acetylcholine chloride 0·005 μg. 3.31 p.m. Fig. 13. Pilocarpine chloride 0·05 μg. 4.20 p.m. Fig. 14. Histamine acid phosphate 0·05 μg. 4.50 p.m. Fig. 15. Unipolar electrical stimulation at *st*. 5.10 p.m. Fig. 16. Oxytocin 0·005 mU. More widespread alveolar contraction and filling of surface duct (*D*). 5.18 p.m.

Figs. 17–24. Repetitive responses showing emptying of part of a lobule into the duct draining it, which is normally hidden beneath alveoli. Drugs applied towards top of field in 0·1 ml. Between responses the alveoli relaxed to the condition shown in Figs. 17 and 19. Note that the alveoli of a neighbouring lobule at the bottom of the field do not all empty, since the glands in this animal were greatly distended. Not all responses are shown. Fig. 17. Control. 2.50 p.m. Fig. 18. Histamine acid phosphate 1 μg. 2.20 p.m. Fig. 19. Control. 3.30 p.m. Fig. 20. Pilocarpine chloride 0·5 μg. 4.55 p.m. Fig. 21. Barium chloride 50 μg. 5.00 p.m. Fig. 22. Acetylcholine chloride 0·01 μg. 5.45 p.m. Fig. 23. Oxytocin 0·1 mU. 6.00 p.m. Fig. 24. Oxytocin 0·1 mU. 6.52 p.m. after atropine sulphate 2 μg at 6.35 p.m. Acetylcholine chloride 0·1 μg at 6.54 p.m. had no effect.