

CAERULEIN SECRETION BY DERMAL GLANDS IN *XENOPUS LAEVIS*

G. J. DOCKRAY and COLIN R. HOPKINS. From the Department of Physiology and the Department of Histology and Cell Biology (Medical), The Medical School, Liverpool University, Liverpool, England

Since gastrin and its related peptides are secreted by a minority population of widely dispersed cells in mammalian tissues it has, in the past, been difficult to study the subcellular aspects of their secretion. From published reports (1, 2) it seemed possible that a satisfactory system for such studies might be provided by the skin of certain amphibians such as *Xenopus laevis* since in these tissues high concentrations of peptides such as caerulein exist, and there is some indication (3) that this, or a similar gastrin-like peptide, may be a dermal gland

secretory product. We have therefore explored this possibility by studying the structure, secretory process, and secretory product of the most prominent nonmucous type of gland in the skin of *X. laevis*. These studies clearly demonstrate that most, if not all, of the caerulein in the skin is contained in secretion granules within the dermal glands and that its release can be specifically evoked by adrenergic stimulation. The release process by a holocrine mechanism expels all of the stored secretion onto the skin surface and thus for

biosynthetic studies it should now be possible to synchronize the processes which lead to the replenishment of the peptide.

MATERIALS AND METHODS

Female *X. laevis* (100–150 g) maintained in tap water were used in all experiments.

Stimulation of Secretion

Adrenaline and noradrenaline in saline were injected directly into the dorsal lymph sac. Electrical stimulation of cutaneous nerves was carried out in animals anesthetized with urethane (1.5 g/kg intraperitoneal) and the nerves were stimulated by platinum electrodes using a stimulator delivering impulses of controlled frequency and voltage.

After stimulation the secretion appearing on the skin surface was collected in either water or saline.

Bioassay of caerulein on the contractions of the gall bladder in urethane-anesthetized guinea pigs was used as a routine biological assay. This assay was introduced for the estimation of cholecystokinin (4) and has been shown to be suitable for the assay of caerulein (5).

The action of caerulein on pancreatic secretion in urethane-anesthetized rats was also used as a bioassay (6). In both the guinea pig and rat bioassays the actions of the skin secretions were compared with those of synthetic caerulein (Farmitalia Ltd., Milan, Italy) and 10% natural porcine CCK (Gastrointestinal Hormone Research Unit, Karolinska Institutet, Stockholm, Sweden).

PURIFICATION OF CAERULEIN: In experiments performed in conjunction with Professor R. A. Gregory a method was devised for the purification of caerulein based on that used to isolate gastrin. The skin secretions of two or three animals were stimulated by injections of adrenaline and collected by immersing the animals in 250 ml saline (0.9%). Saline containing the secretory product was removed, and poured into 10 liters boiling water. The solution was then briefly boiled (2–3 min) to inactivate proteolytic enzymes and disrupt secretory granules. The solution was cooled and the precipitate which formed was removed by filtering through fiber glass paper and was then discarded. Diethylaminoethyl-cellulose (DEAE; Whatman 11, 5 g), pretreated by the methods recommended by the manufacturers, was added to the solution and stirred for 15 min. The solution was again filtered and the DEAE collected on a Buchner funnel. Biological assay of the filtrate showed that 98–99% of the caerulein had been taken up by the DEAE. The caerulein was eluted from the DEAE with 1.0 M ammonium carbonate (50 ml), and this solution was freeze dried. The purification of caerulein from this material was achieved by chromatography on small columns of aminoethylcellulose (AE).

The amino acid composition of purified caerulein was

determined after hydrolysis of samples with either 6 N HCl at 110°C for 18 h, or 5 N NaOH at 105°C for 20 h in sealed vacuum tubes. The amino acid analyses were carried out in a JEOL automatic amino acid analyser (JEOL Analytical Instrument Div., Cranford, N. J.) (J. L. C. 5A. H.).

Light and Electron Microscopy

Thin strips of skin (3–4 mm wide) were fixed in formol saline and processed routinely for light microscopy. For electron microscopy 1–2-mm wide strips were fixed in Karnovsky's glutaraldehyde-paraformaldehyde fluid (7) 3 h at room temperature, postfixed in 1% osmium tetroxide (phosphate buffered to pH 7.4), stained en bloc with uranyl acetate (8) for 45 min, dehydrated, and embedded in Epon (9). Sections were cut on a Huxley ultramicrotome mounted on naked grids and examined in a Siemens Elmiskop I. Released secretion was collected in either water or saline, pelleted at 10,000 g in a Beckman microfuge for 5 min (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and then fixed and embedded like the strips of skin.

RESULTS

In the initial experiments an attempt was made to detect and assay caerulein in the secretion released from the nonmucous 'granular' glands. The experiments were based on preliminary observations, described more fully below, which indicated that of the various kinds of subepidermal glands in the skin of *X. laevis* only the granular glands being studied responded to adrenergic secretagogues.

Caerulein and Glandular Secretion

Secretion collected from the skin of adrenaline-injected individuals strongly stimulated contractions of the guinea pig gall bladder *in situ*. In contrast, secretion from the skin of uninjected or saline-injected animals possessed relatively weak activity on the gall bladder (Fig. 1). The responses of the gall bladder to the adrenaline-induced skin secretions closely resembled those to synthetic caerulein and natural cholecystokinin (Figs. 1 and 2). In Fig. 2 it can be seen that a fraction as low as 0.00005% of the secretion produced by a single animal after stimulation with adrenaline (100 $\mu\text{g}/\text{kg}$) stimulated gall bladder contraction. Using synthetic caerulein as a standard, the mean total quantity of caerulein secreted by each of the five animals (mean weight 120 g) after adrenaline stimulation (100 $\mu\text{g}/\text{kg}$) was estimated to be 14.1 ± 2.9 mg.

Skin secretion from *X. laevis* also stimulated

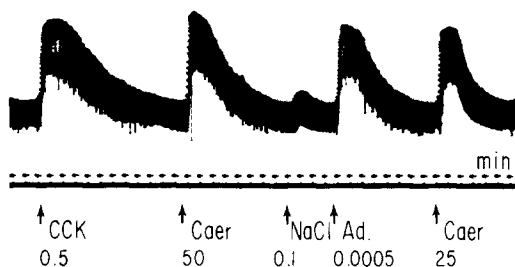


FIGURE 1 Responses of the gall bladder in a urethane-anesthetized guinea pig (550 g) to CCK, synthetic caerulein, and secretion from the skin of *X. laevis*. Contractions of the gall bladder are shown by an upward deflection of the trace. Skin secretions were collected from a single 120-g animal after the injection of either saline (0.2 ml) or adrenaline (25 $\mu\text{g}/\text{kg}$). CCK, cholecystokinin; dose is in Ivy dog units. Caer, synthetic caerulein; dose is in nanograms. NaCl, secretion of *X. laevis* collected after injections of saline; dose is given as a percentage of the total secretion collected. Ad, secretion collected from the same animal after stimulation with adrenaline; dose is given as a percentage of the total secretion.

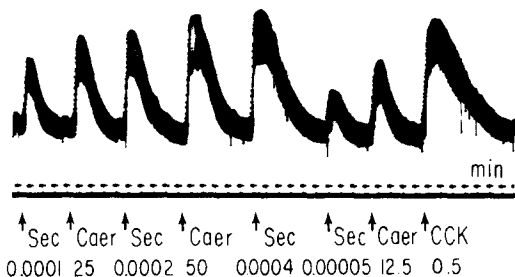


FIGURE 2 Comparison of the action of various doses of synthetic caerulein and adrenaline-stimulated secretion on the gall bladder of the guinea pig. Experimental conditions are as in Fig. 1. Caer, caerulein; dose is in nanograms. Sec, skin secretion collected after the injection of adrenaline (100 $\mu\text{g}/\text{kg}$); dose is given as a percentage of the total secretion.

pancreatic secretion in the rat. Fig. 3 shows that synthetic caerulein strongly stimulated both the rate of flow and rate of protein secretion from the rat pancreas, and that the responses to injections of adrenaline-induced skin secretion were similar. From its activity in the rat, the sample of secretion tested in the experiment shown in Fig. 3 was estimated to contain 2.3 $\mu\text{g}/\text{ml}$ caerulein, while in the guinea pig the same sample of secretion was estimated to contain 3.5 $\mu\text{g}/\text{ml}$.

Further information on the identity of the active factor in the adrenaline-stimulated secretion was sought from its isolation by using column chroma-

tography on AE. Fig. 4 shows a typical elution profile obtained with a sample of partially purified secretion. Four peaks with optical density at 280 nm emerged from the column, and caerulein-like biological activity was associated mainly with peak II but to a lesser extent with peak IV also. Fractions corresponding to peak II were pooled,

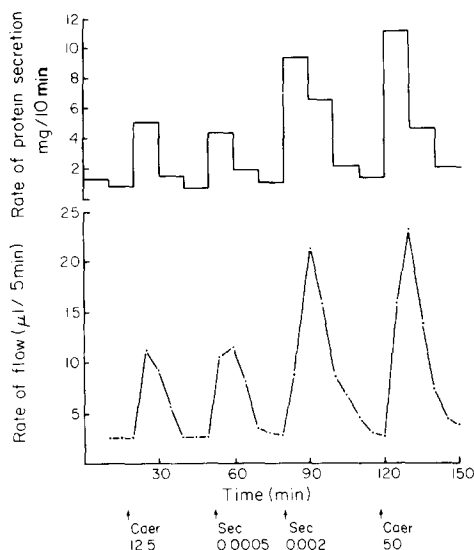


FIGURE 3 The response of the pancreas in a urethane-anesthetized rat (245 g) to synthetic caerulein (Caer), and to adrenaline-stimulated secretion (Sec). Secretion collected from single 130-g animal after injection with adrenaline (25 $\mu\text{g}/\text{kg}$).

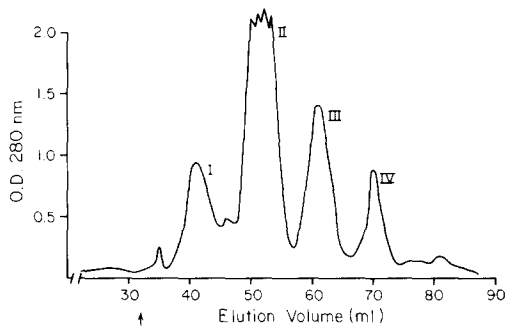


FIGURE 4 Elution of partially purified skin secretion from an aminoethylcellulose (Whatman AE 41, 1×16 cm) column. The sample was applied in 0.025 M ammonium bicarbonate, and the column was washed with this buffer until the eluate was free of protein. The gradient from 0.025 M (arrow) to 0.5 M ammonium bicarbonate was then run. Volume of mixing vessel: 150 ml. Fraction size: 1.0 ml; fractions collected every 15 min.

freeze dried, and rerun on a smaller column (1×5 cm) of AE under conditions similar to those of the first run. A single, well resolved peak containing biological activity emerged. The active fractions from the rerun eluate were again pooled and freeze dried and the amino acid composition of the sample was analyzed. The purified secretion was 1.15 times more potent than synthetic caerulein in stimulating gall bladder contractions in the guinea pig.

The amino acid analysis for peak II material is shown in Table I. It can be seen that the composition of this material, after hydrolysis with 6 M HCl, closely resembled that of caerulein. During acid hydrolysis the tyrosine-*O*-sulfate linkage is broken, so that an estimation of this residue in caerulein is not possible. This change does not occur in alkaline hydrolysis, however, and it can be seen that, after hydrolysis with NaOH, tyrosine-*O*-sulfate was identified and estimated to be present in peak II material in quantities similar to those found in caerulein. Acid hydrolysis also destroys the tryptophan residue, but the presence of this amino acid in the purified caerulein was apparent from the UV absorption spectra of the sample. The material in peak IV has also been found to have an amino acid composition similar in some respects to that of caerulein, but further work is needed to establish the identity of this material.

Structure of the Granular Glands

The dermal layers of the dorsal skin in *X. laevis* contain several kinds of glandular structure

TABLE I
Amino Acid Composition* of Peak II Material
Relative to Glycine (1.00)

Amino acid	Composition of caerulein	Composition of peak II	
		Acid hydrolysis	Alkaline hydrolysis
Aspartic acid	2	2.06	2.10
Threonine	1	0.92	0.85
Glutamic acid	2	2.31	2.10
Glycine	1	1.00	1.00
Methionine	1	0.82	0.90
Phenylalanine	1	0.92	0.92
Tyrosine-sulfate	1	-	0.80
Tyrosine		0.97	-
Tryptophan	1	-	-

* No amino acids other than those shown were present in concentrations greater than 0.02.

opening via epidermal ducts to the external surface. However, mucous glands and the slightly larger, granular glands predominate, and these two kinds of glands are present in approximately equal numbers. There are no overt structural changes in the mucous glands after stimulation with adrenaline (Figs. 6 and 7), and we therefore assume that the induced secretion is derived primarily from the granular glands.

The granular glands have the general organization shown in Fig. 5. The fine structural organization of the myoepithelial cell envelope is similar to that found in other types of gland (10) and

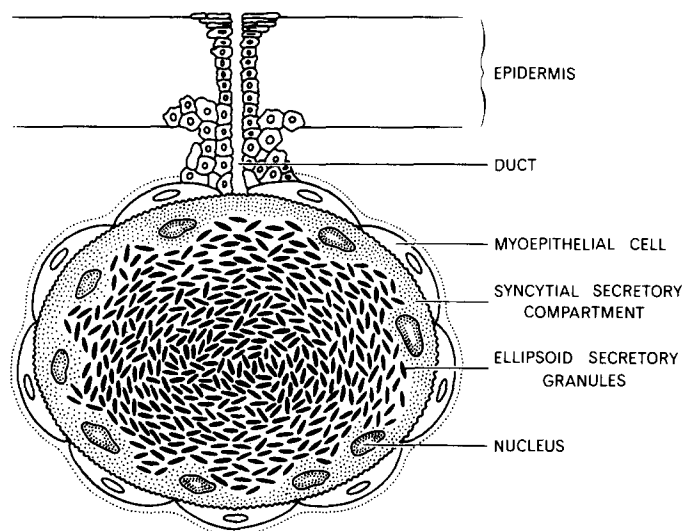


FIGURE 5 Schematic representation of a cutaneous granular gland in *X. laevis*.

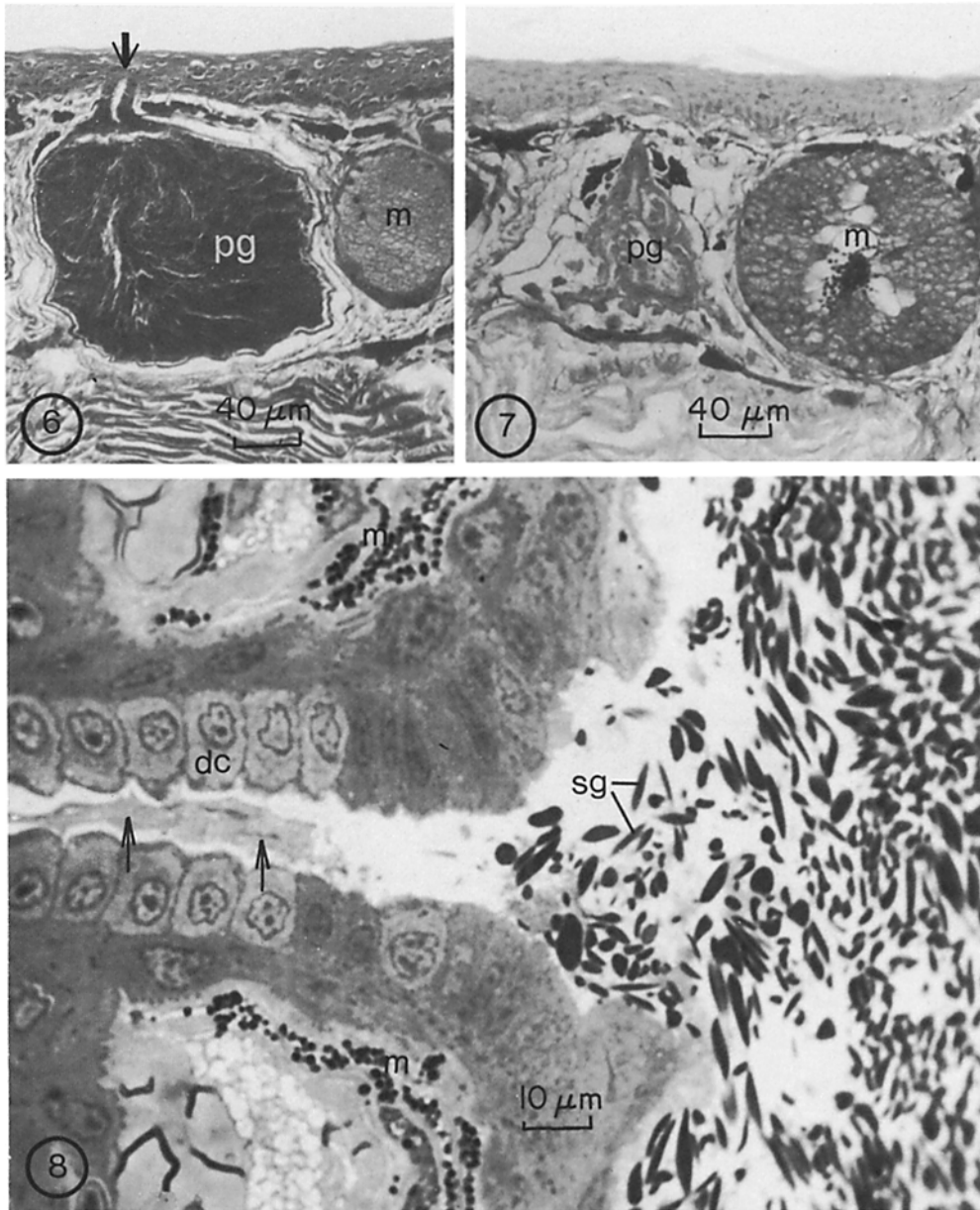


FIGURE 6 Unstimulated granular gland (*pg*) showing position of the duct (arrow) and the densely stained content. A typical mucous gland (*m*) is shown in tangential section. Paraffin section, periodic acid-Schiff, light green staining. $\times 250$.

FIGURE 7 Contracted gland (*pg*) in the skin of an animal injected with $50 \mu\text{g}/\text{kg}$ adrenaline 5 min previously. The content has been entirely expelled. Mucous gland (*m*) in sagittal section shows no overt morphological change following the injection. Paraffin section, periodic acid-Schiff, light green staining. $\times 250$.

FIGURE 8 Skin stimulated with $50 \mu\text{g}/\text{kg}$ adrenaline and then fixed immediately. The secretory product is being released, and the section shows the lumen of the gland duct and part of the gland syncytium. The duct contains amorphous material derived from degraded secretory granules (arrows), while deeper in the gland intact secretory granules (*sg*) remain. *dc*, duct cells; *m*, melanocytes. Epon section, Karnovsky fixation, methylene blue/azure II staining. $\times 960$.

shows no special features. The secretory compartment is a syncytium within which secretory granules occupy the central cytoplasmic area, whereas the nuclei, which are surrounded by well developed cisternae of rough endoplasmic reticulum and elaborate Golgi complexes, are located at the periphery (Fig. 9). The secretory granules are spheroidal, membrane-bounded and in many cases display an underlying longitudinal periodicity within their electron-opaque content (Figs. 9, 14, 15). In the Golgi area frequent profiles are encountered which suggest that secretory granules arise by the fusion of vacuolar structures derived from the Golgi complex.

The plasma membrane surrounding the gland forms small irregular microvilli, and it is continuous across the opening at the base of the duct. Between the plasma membrane of the secretory compartment and that of the myoepithelial cell, nerve fibers, and less commonly nerve fiber endings, are seen (Figs. 10 and 11). There is no obvious thickening of the pre- or postsynaptic membranes in these endings, but their content of numerous, small synaptic vesicles (mean diam. 500 Å) with electron-translucent contents and fewer larger (mean diam. 1500 Å) vesicles with central electron-opaque inclusions indicates that they are adrenergic in nature (11).

Stimulation of Glandular Secretion

Electrical stimulation of the main nerve trunks (15 V; 50 cycle/s) to the skin of the dorsal surface produced an immediate discharge of milky, viscid secretion from pores on the surface of the skin. Injection of adrenaline or noradrenaline via the dorsal lymph sac, or directly into the subepidermal tissue, evoked a similar response within 30 s and, once initiated, the secretion continued for 2–3 min. Thereafter, repeated stimulation failed to evoke any further secretion.

The time courses of the responses were also followed morphologically. Thus skin 'fixed' 30 s after stimulation by a close intradermal injection of adrenaline showed that about 60% of the glands within the immediate area had started to release their content. When the skin was fixed 5 min after injection, it was apparent that almost all the glands had discharged. Extraction of the skin with 80% methanol (1) at this time showed that 80–90% of the caerulein activity obtainable from unstimulated skin had been secreted.

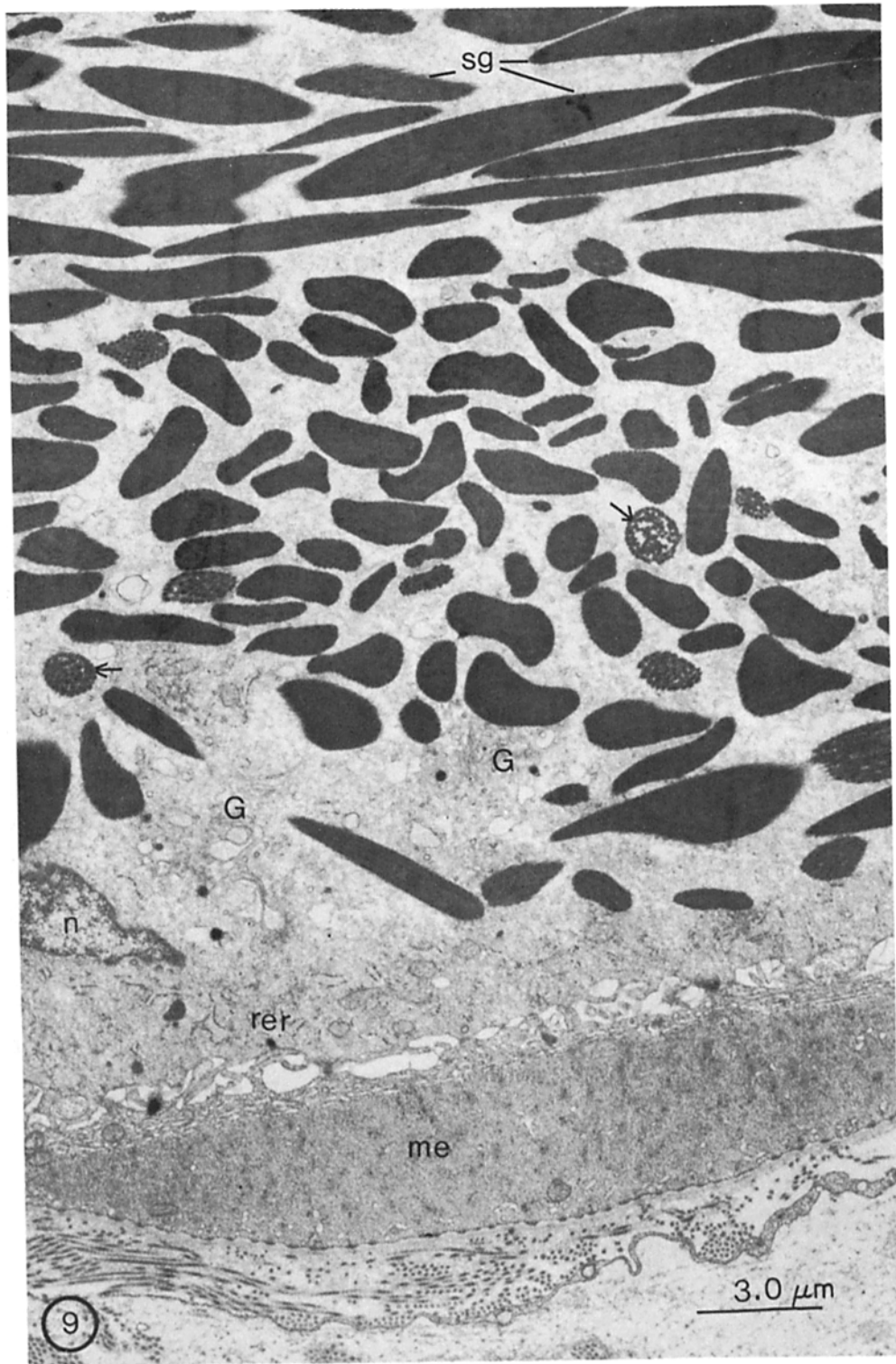
The threshold dose of adrenaline or noradrenaline which stimulated the discharge of granular glands was 1–2 µg/kg. Doses close to the threshold only stimulated secretion from glands around the site of injection, but with larger doses, progressively greater areas of skin were stimulated. Thus a dose of 50 µg/kg usually evoked a copious secretion from the entire dorsal surface, although in glands distal to the site of injection the response was often delayed for a period of 2–4 min.

The Release Process and the Nature of the Secretory Product

The release of secretory product is a rapid and disruptive event. Presumably it occurs as a result of the contraction of the myoepithelial cell envelope compressing the secretory compartment and inducing the plasma membrane at the base of the gland duct to rupture. The contents of the gland are then expelled onto the skin surface (Fig. 8), although most of the nuclei and some of the peripheral cellular elements remain within the gland. After secretion, reconstitution and not replacement of the gland occurs. To reach the stage where adrenaline will again stimulate a discharge takes approximately 2 wk.

The manner in which the secretory granules are released suggests that, unlike most other release mechanisms (12), these granules should retain their enveloping membranes on release. To investigate this possibility, secretion was examined which had been collected either in saline or in water.

When the secretion which had been collected in saline was centrifuged (10,000 g for 5 min), the pellet produced contained 50–80% of the total caerulein activity. Fixed preparations of these pellets showed that they contain predominantly membrane-bounded granules, most of which were morphologically identical to those observed within the unstimulated gland (Figs. 12 and 13). Among the released granules, however, there were always a number which, although membrane bounded, appeared less electron opaque (Fig. 14). Their appearance may well reflect some leakage of their content which would account for the variable proportion of caerulein activity recovered in the supernate. In contrast, when the secretion was collected in water and centrifuged, all of the caerulein activity was present in the supernate. Under these conditions the pelleted material had a finely fibrillar appearance, and there were no granules or granule-like particles present.



DISCUSSION

Anastasi et al. (1) described the isolation of a decapeptide, caerulein, from extracts of the skin of the Australian tree frog, *Hyla caerulea*, and subsequently, caerulein was found in extracts of the skin of other species of amphibia including *X. laevis* (2). There are remarkable similarities in structure between the C-terminal portions of caerulein and the two mammalian hormones gastrin and cholecystokinin (CCK). In particular, caerulein and CCK share the same C-terminal heptapeptide (with the exception of a single substitution of methionine in CCK for threonine in caerulein), and their C-terminal pentapeptides are identical with those in gastrin. Since the C-terminal heptapeptide of CCK is the minimal fragment of the molecule possessing strong activity on the guinea pig gall bladder (13) and probably on the rat pancreas also (14), it is not surprising to find that caerulein is highly active in these two preparations (5, 6).

The present study has shown that secretion collected from the external surface of *X. laevis* after an injection of adrenaline has actions on the gall bladder and pancreas closely resembling those of synthetic caerulein. Moreover, the active factor in the secretion is a peptide with an amino acid composition identical to that of caerulein. Together, these results clearly demonstrate that caerulein is one of the components of dermal gland secretion.

Under resting conditions there is little or no demonstrable secretion of caerulein. However, after injections of adrenaline into the dorsal lymph sac or stimulation of the cutaneous nerves, there is prompt and copious secretion and an almost total discharge of the cutaneous granular glands. The mucous glands remained overtly unchanged. The granular glands in *X. laevis* are characterized by the presence of a large number of densely packed granules lying in a syncytium, with nuclei placed peripherally, and surrounded by myoepithelial cells. Contraction of myoepithelial cells is presumed to force the granules and other organelles

along the gland duct and onto the external surface. When the secretion was collected in saline, intact granules were obtained, and these granules were found to be associated with a major part of the total caerulein activity in the expelled secretion. These results demonstrate that caerulein originates in the cutaneous granular glands and that it is stored in the granules found within these glands.

Our finding that adrenergic nerve endings are present at the junction between the myoepithelial cell envelope and the secretory compartment supports the view that the discharge of the granular glands is under direct nervous control and provides a morphological basis for the interpretation of previous studies that secretion by dermal glands in *X. laevis* is stimulated by adrenaline (15-17).

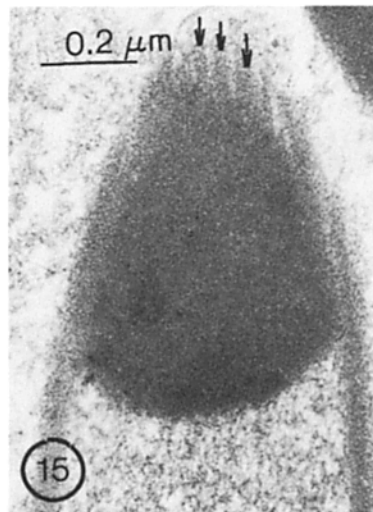
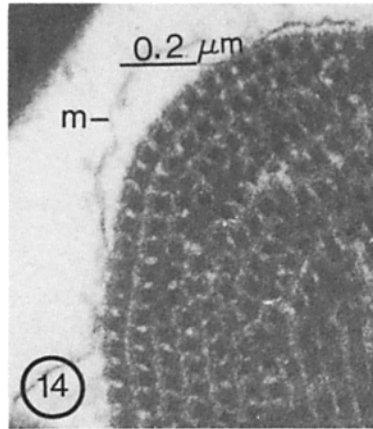
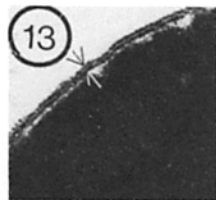
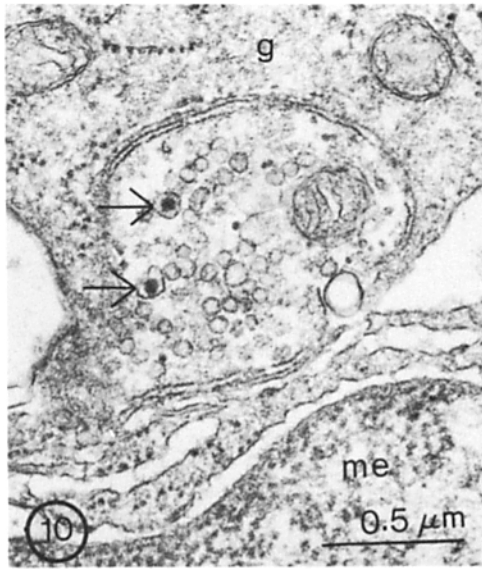
The secretory process in the dermal granular glands warrants further and more detailed study. It is of interest in that the release of secretory product does not involve exocytosis, and consequently, we have been able to collect and assay a virtually intact secretory granule population. This feature is particularly advantageous in the use of this system as a model in investigations of the biosynthesis and secretion of a small peptide. Moreover, for studies of biosynthesis alone the system also offers the opportunity of depleting the glands and thus synchronizing the processes of restoration and the replenishment of the stores of secretory product.

SUMMARY

Cutaneous granular glands in *X. laevis* secrete the gastrin-like peptide caerulein in response to adrenergic stimulation. After their release by a holocrine mechanism, the secretory granules from these glands can be collected intact and shown to contain caerulein.

We are indebted to Professor R. A. Gregory for encouragement and discussion throughout this work and for help in the purification of caerulein from skin secretion. Mr. Barry Mason (Department of Organic Chemistry, University of Liverpool) very kindly performed the amino acid analyses. Synthetic caerulein was a gift from

FIGURE 9 Electron micrograph illustrating the periphery of the unstimulated gland. Part of the myoepithelial cell envelope (*me*) which surrounds the syncytial glandular portion is shown. Within the glandular portion a nucleus (*n*), Golgi complexes (*G*), cisternae of rough endoplasmic reticulum (*rer*) and mature secretory granules (*sg*) are present. The more peripheral secretory granules are cut in transverse or oblique/transverse section, and some of them (arrowed) are partially extracted revealing a rodlike substructure. $\times 7,000$.



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FIGURE 10 Nerve ending situated between the myoepithelial cell envelope (*me*) and the glandular compartment (*g*) in tissue from an unstimulated animal. Small, synaptic (500-Å diam.) vesicles with an electron-translucent content and larger vesicles (1,500-Å diam.) with electron-opaque cores (arrows) are present within the ending. $\times 40,000$.

FIGURE 11 Nerve ending found between myoepithelial cell envelope and glandular compartment in tissue from a stimulated (50 µg/kg adrenaline) animal. Although the tissue is highly disorganized at this time, the innervation remains intact. *ax*, axon; *m*, mitochondrion. $\times 40,000$.

FIGURE 12 Electron micrograph of granules collected from secretion expelled into saline. $\times 13,000$.

FIGURE 13 The same preparation as shown in Fig. 12 illustrating the unit membrane (arrows) which surrounds the granules within the gland and which remains intact when the secretion is expelled into saline. $\times 150,000$.

FIGURE 14 Partially extracted granule similar to those arrowed in Fig. 9 illustrating the substructure of the granule content sectioned transversely. *m*, limiting membrane of the granule deflected away from the granule surface. $\times 50,000$.

FIGURE 15 Portion of a maturing secretory granule sectioned obliquely showing the forming rodlike substructure (arrows) in longitudinal section. $\times 60,000$.