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The Sequestration, Processing and Retention of Honey-Bee Promelittin Made in Amphibian Oocytes

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Messenger RNAs from one kind of secretory cell can be introduced into the cytoplasm of another: the heterologous proteins formed by the recipient cell are usually processed and topologically segregated in the manner characteristic of the donor cell. *Xenopus* oocytes injected with honey-bee venom gland RNA provide some support for this generalization, but also reveal important exceptions to it. Thus, the frog cell makes a small polypeptide whose partial sequence matches perfectly that of insect promelittin, except that the product formed in oocytes ends at the C terminus with a glycine as opposed to a glutamine amide residue. N-terminal heterogeneity is seen in protoxin made in oocytes and venom gland cells, and species shorter by two residues are seen in both tissues. We suggest that the oocyte contains a dipeptidylpeptidase. Promelittin made in oocytes is barely detectable in the cytosol but is found associated with a vesicle fraction which also contains some newly synthesized endogenous oocyte proteins. The association with vesicles is long-lasting; thus promelittin is retained slightly more efficiently than sequestered oocyte proteins, and an incubation period of about two weeks is required to reduce by half the amount of these endogenous vesicle proteins. Thus neither promelittin nor any products derived from it are secreted rapidly. Gel analysis fails to reveal promelittin in the medium surrounding the oocyte, although traces can be detected by assaying for a characteristic heptapeptide. Such small amounts could result from slow secretion or leakage. Melittin could not be detected by gel analysis or peptide assay. The retention of the honey-bee protein within the frog cell is discussed in terms of the specificity of the processing systems and secretory pathways of venom gland cells and oocytes. We suggest that whilst some export mechanisms function efficiently in a wide variety of cells, others do not, and may even be restricted to specific cell types.

Proteins characteristic of one kind of cell can be synthesized in the cytoplasm of another [1–4]: their fate can reveal the nature and specificity of the post-translational machinery [5]. Moreover, the factors affecting the subcellular localization of newly made proteins can be studied by fractionation of cells making such heterologous proteins. The microinjection of secretory protein messengers into *Xenopus* oocytes shows the presence of a secretory pathway [6–8], which is selective but appears to lack both species and cell-type specificity. Detailed studies require knowledge of the precise structure of both the newly made secretory protein and its processed forms. Honey-bee prepromelittin meets these requirements for the amino acid sequence has been determined [9] and, for many parts of the molecule, structure has been correlated with function. Thus four different regions, starting from the amino end, have been identified in the primary translation product; as shown in Fig. 1 there is a pre or signal sequence of 21 residues, a pro part comprised of 22 amino acids [10], the 26 residues of melittin [11] and, finally, an extra glycine residue [9]. Several enzymic reactions are required to convert prepromelittin to melittin. The first, the cleavage of the pre sequence [12], is probably a co-translational event which can, however, also be detected *in vitro*, in the presence of rat liver microsomes, in a post-translational assay [13]. In the second step the extra glycine is removed with concomitant formation of the carboxy-terminal amide [10]. Finally promelittin must be activated to form the toxin melittin. This latter reaction proceeds by the stepwise action of a dipeptidylpeptidase IV [14,15], which cleaves after proline or alanine residues [15a]. Thus the oocyte injected with venom gland RNA [16] provides a useful system for studying the synthesis and fate of a heterologous secretory protein, for prepromelittin and its processed forms are small, well-defined polypeptides that undergo substantial cleavages and interesting modifications.

MATERIALS AND METHODS

Handling of Oocytes

Oocytes were injected [1] with honey-bee venom gland RNA [16] and then cultured in modified

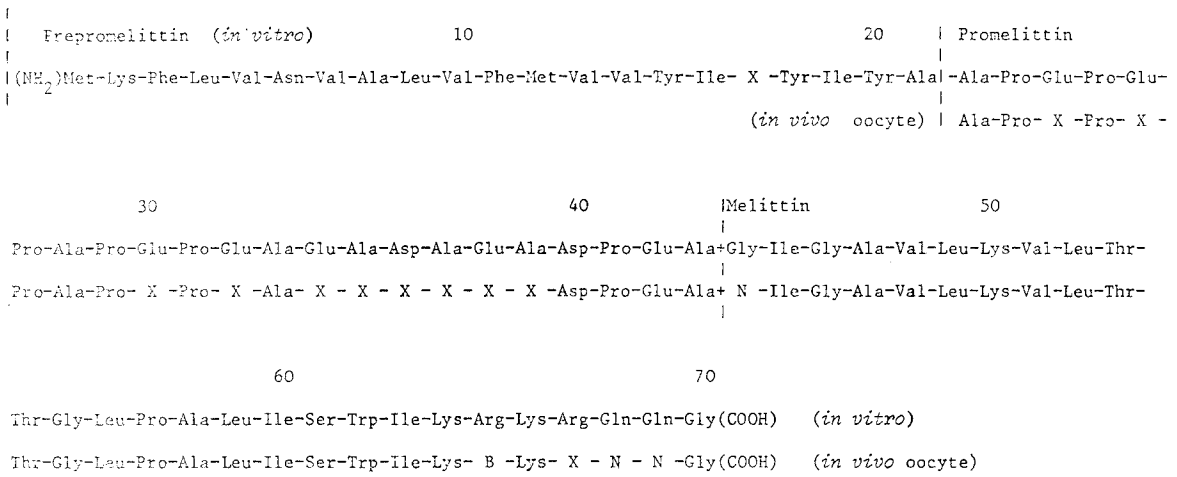

Fig. 1. *The partial sequence of promelittin made in oocytes under the direction of venom gland RNA.* The sequence of prepromelittin [9] made in a wheat germ system is shown for comparison. RNA-injected oocytes were labelled separately with [^{3}H]valine, [^{3}H]proline, [^{3}H]leucine, [^{3}H]isoleucine, [^{3}H]serine, [^{3}H]threonine, [^{3}H]alanine, [^{3}H]tryptophan or [^{3}H]glycine. Promelittin was purified and fragments were sequenced. The sequence information for the oocyte product was obtained both as described in the present and as in a previous [16] publication. It should be noted that promelittin and melittin formed in venom gland cells and with the C-terminal structure Gln-Gln-NH_2 [21]. In Fig. 1 unidentified residues are denoted by the symbol (X), basic ones by (B) and neutral residues by the symbol (N)

Barth X medium [17] containing additional antibiotics (penicillin 100 units/ml, streptomycin 100 µg/ml, gentamycin 70 µg/ml, mycostatin 20 units/ml). After 24 h incubation, which permits both messenger recruitment [18] and selection of intact oocytes, radioactive amino acids ([^{3}H]alanine, [^{3}H]valine, [^{3}H]leucine, [^{5}H]proline, [^{3}H]isoleucine, [^{3}H]serine, [^{3}H]-threonine, [^{3}H]tryptophan and [^{3}H]glycine, 0.5–5.0 mCi at 1–10 mCi/ml and >10 Ci/nmol) were added. Chase conditions were established [18] by transferring oocytes to unlabelled medium and, 1–4 h later, adding the appropriate amino acid (5–10 mM).

Biochemical Analysis

After removal of the incubation medium, oocytes were fractionated into cytosol, yolk and vesicle contents [8]. Aliquots of these cells fractions were analyzed on 17.5% dodecyl sulphate gels [19] and radioactive polypeptides were visualized by fluorography [20]. Under these conditions promelittin usually migrates as a diffuse doublet, with a mobility somewhat greater than that of globin. Promelittin present in whole oocytes, subcellular fractions or the incubation medium was isolated by extraction with butan-1-ol followed by descending chromatography of the aqueous layer, a method first applied to isolation of the protoxin from the venom glands of honey-bees [21]. After extraction, melittin is found in the butanol phase, whilst larger proteins, most of which are denatured, accumulate at the interface. Promelittin used for Edman degradation was eluted from the chromatogram and further purified by paper electrophoresis at pH 4.8 (1% pyridine/acetate, Whatman 3MM paper). Under these conditions most of the promelittin remains at the origin but a portion migrates just away from the origin towards the anode. This material (both portions) was again eluted, subjected to manual Edman degradation and the butyl-acetate-extractable radioactivity was measured after each step. However, in most experiments involving sequencing studies, promelittin was first digested with chymotrypsin (50 µg enzyme/0.25 ml incubation medium at 30 °C for 3 h at pH 8). Fractionation of the resulting digest by paper electrophoresis at pH 4.8 yields, amongst other peptides, a large acidic fragment, which contains the entire pro region as well as the NH_2-terminal hexapeptide of melittin (i.e. residues 22–49 of prepromelittin [9]) and which has a mobility about half that of glutamic acid. This fragment can be further purified by paper electrophoresis at pH 1.8 (10% acetic acid adjusted with formic acid) and can then be sequenced by stepwise Edman degradation.

Whole oocytes, subcellular fractions and incubation media were all analyzed by the same procedures. In experiments with [^{3}H]proline, the large acidic fragment was isolated from chymotryptic digests of different chromatographic fractions and the amount of promelittin was deduced from the total radioactivity extractable after the second stop of Edman degradation. This corresponds to the first proline residue in the fragment, whether this is derived from a full-length promelittin species or from species shorter by two or four residues. Uninjected controls were analyzed in parallel; thus the same electrophoretic fractions were eluted and the radioactivity extracted in the second Edman step was used to establish the level of background radioactivity. In experiments with labelled leucine, valine or glycine, promelittin was isolated by the same chromatographic

procedure, but was then digested with pepsin and fractionated by paper electrophoresis at pH 4.8. This yields the basic heptapeptide Lys-Val-Leu-Thr-Thr-Gly-Leu, which comprises residues 7–13 of the melittin region. Radioactivity recovered in this fragment, again after subtraction of the value obtained with the uninjected control, also yields the amount of radioactive promelittin present, given the known sequence of the whole protein.

In several experiments the organic layer obtained after extraction with butan-1-ol was analyzed for the possible presence of melittin. In these studies the butanol phase was dried, digested with pepsin, fractionated by paper electrophoresis and then monitored for the presence of the basic heptapeptide (melittin 7–13).

The carboxy-terminal region of oocyte promelittin was analyzed by tryptic cleavage of a C-terminal fragment obtained by electrophoresis at pH 4.8 of a pepsin digest: details of the approach are as described for prepromelittin [9]. In general, sequencing studies followed procedures already applied to venom gland, oocyte or *in vitro* products [9, 16, 21, 22].

RESULTS

Structure and Heterogeneity at the Amino-Terminus of Promelittin Made in Oocytes

Oocytes were injected with venom gland RNA and incubated with either [^{3}H]proline or [^{3}H]alanine. Intact promelittin or the large fragment isolated from chymotryptic digests was used for manual Edman degradation; the amount of radioactivity that could be extracted by butyl acetate was measured after each step. Thus the amino-terminal sequence of oocyte promelittin was shown to be:

Ala-Pro-X-Pro-X-Pro-Ala-Pro-X-Pro-X-Ala- . . .

This is identical to the amino-terminal sequence of promelittin isolated from the venom glands of queen bees which had been injected with radioactive amino acids. Promelittin from both sources thus starts at residue 22 of prepromelittin synthesized *in vitro* [10].

In most, but not all experiments we detected a promelittin species shorter by two residues at the amino terminus. Two such experiments are presented: thus Fig. 2A shows paper electrophoresis of a chymotryptic digest of [^{3}H]proline-labelled promelittin. A large chymotryptic fragment is present in fractions 12–14. The main peak, found in fraction 12, and two minor peaks in fractions 13 and 14, were eluted and subjected separately to Edman degradation. As can be seen from Fig. 3A, fraction 12 yields the expected pattern with proline residues present at positions 2, 4, 6, 8 and 10. The material present in fractions 13 and 14 gave, however a different pattern (see Fig. 3B) with

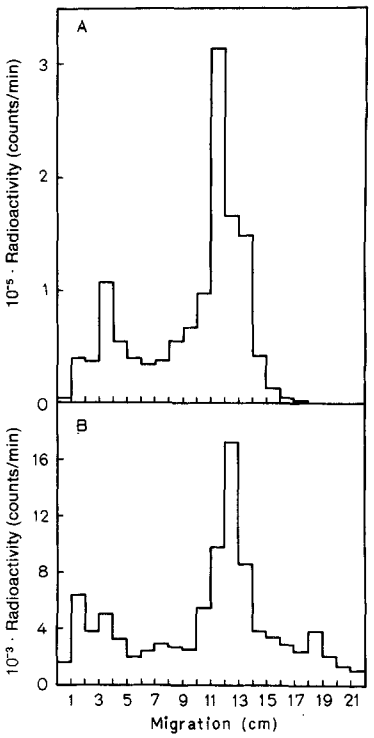

Fig. 2. *(A) Paper electrophoresis of a chymotryptic digest of proline-labelled promelittin made in oocytes. (B) Similar experiment performed with oocytes labelled with [^{3}H]alanine for 24 h.* Frog cells were injected with venom gland RNA and incubated in [^{3}H]-proline for 2 days. Promelittin was prepared by chromatography of the aqueous phase following butanol/water extraction of the labelled oocytes. A peak of proline-labelled material with the mobility of honey-bee promelittin, was present in RNA injected but not in control oocytes. This heterologous translation product was eluted from the chromatogram, digested with chymotrypsin and then electrophoresed at pH 4.8: radioactive material (——) in (A) was eluted and counted. A fragment of the same mobility as the 29-amino-acid prepromelittin chymotryptic peptide (residues 21–49) is seen in material from mRNA-injected (A) but not control oocytes (data not shown)

only four alternating proline residues: the fifth residue could not be detected. Similar results have been obtained in two further experiments performed with oocytes obtained from separate frogs and injected with different RNA preparations. In each instance the fifth proline residue was missing from the part of the peak migrating faster at pH 4.8 and/or at pH 1.8, where a fragment shorter by two residues but with the same net charge would be expected to run. Thus artefacts resulting from the Edman degradation or the condition of the oocytes are rendered unlikely.

A similar experiment performed with promelittin containing [^{3}H]alanine is shown in Fig. 2B, 3C and D. Again the slower and faster-migrating regions of the large chymotryptic fragment shown in Fig. 2B were analyzed separately. The slower fragment yielded alanines in positions 1 and 7 (Fig. 3C) as expected for the full-length species. The faster peptide gave a more complex pattern (Fig. 3D), which can be inter-

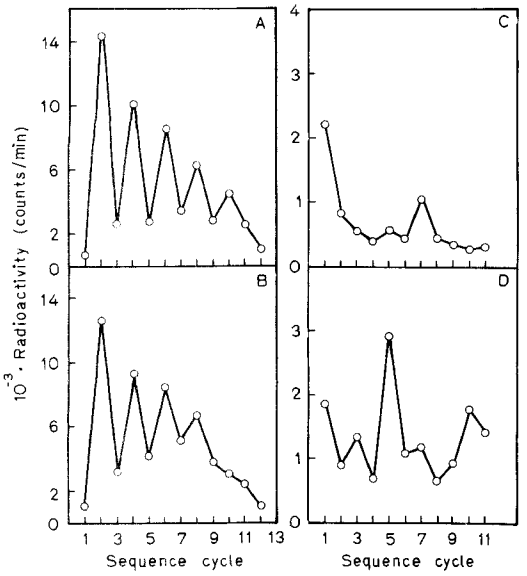

Fig. 3. *Sequential Edman degradation of large chymotryptic fragments from oocyte-derived promelittin.* (A, B) [^{3}H]Proline-containing, (C, D) [^{3}H]alanine-containing material. Thus large chymotryptic peptides were purified by electrophoresis at pH 4.8: slow-moving (fraction 12 of Fig. 2) and fast-moving (fractions 13 and 14 of Fig. 2) fragments were eluted and subjected, separately, to sequential Edman degradation. The radioactivity released after each cycle was measured, and plotted against the number of rounds of degradation. (A, C) The pattern obtained with the slower-moving peptides; (B, C) that of the faster-moving material

preted as follows: some of the fragments present in this fraction contain alanine in positions 1 and 7, but the main component clearly has alanine in positions 5 and 10 and is, therefore, shorter by two residues. There is even a small peak of radioactive alanine in position 3, which could originate from a peptide shorter by four residues, but we have no further data to support this idea.

Studies on the Incorporation of [^{3}H]Glycine: the Carboxy-Terminal Sequence of Oocyte Synthesized Promelittin

Promelittin extracted from venom glands terminates with the sequence Gln-Gln-NH_2, yet prepromelittin made in the wheat germ cell-free system has a free α-carboxyl group [22] at its C terminus and the structure Gln-Gln-Gly. Thus it was of interest to establish the sequence of the processed product made in oocytes under the direction of venom gland RNA. These experiments were hampered by the poor incorporation of [^{3}H]glycine into promelittin, despite a relatively small pool size [23]. It seems that a substantial fraction, as much as one-third, of the added label is incorporated into a substance which upon electrophoresis at pH 3.0 shows the mobility expected of a γ-glutamic acid peptide. On reduction this substance migrates with reduced glutathionine: it is found in large amounts both inside and outside the oocyte, as might be expected from observations made with mammalian cells [24]. Nonetheless, evidence for a glycine residue at the carboxyl end of oocyte promelittin could be obtained using the experimental strategy described for prepromelittin [22].

Thus premelittin was digested with pepsin and the hydrolysate was fractionated by paper electrophoresis at pH 4.8. Such a procedure yields a basic fragment, containing radioactive glycine, of mobility about 0.75 relative to free lysine. This peptide, which is known to be derived from the carboxyl end, was eluted and digested with trypsin. Electrophoresis now yielded a neutral peptide, which was shown by manual Edman degradation to have glycine in the third position. It is, therefore, very likely that oocyte promelittin has the same carboxy-terminal structure as prepromelittin synthesized *in vitro* in the absence of added membranes. As shown in the next section, newly made promelittin is transferred across or into endoplasmic reticulum of the frog cell. Nonetheless oocytes are unable to remove the terminal glycine residue and form the amidated glutamine moiety. In the course of these and earlier experiments [16], the sequence of 34 of the 49 residues of oocyte-synthesized promelittin has been determined (see Fig. 1). The partial sequence of the frog cell product matches perfectly that of insect promelittin, except of course, for the absence in the latter of the C-terminal glycine residue.

The Subcellular Distribution of Promelittin made in Oocytes

Oocytes injected with venom gland RNA were incubated in [^{3}H]valine and then fractionated into supernatant (cytosol), detergent-solubilized vesicles (DS1) and yolk pellet. Dodecyl sulphate gel analysis reveals a species, absent from control oocytes, with the mobility expected for promelittin: as shown in Fig. 4 this heterologous polypeptide is present in large amounts in the vesicle extract, but only traces can be found in the cytosol, which nonetheless contains over 80% of the newly made protein. The presence of promelittin in the vesicle fraction was confirmed by electrophoretic analysis of peptic digests, which revealed the characteristic heptapeptide (melittin 7–13). The transfer of a newly synthesized insect polypeptide across or into the membranes within a frog cell shows that the systems involved lack even phylum specificity.

Changes in the subcellular distribution of promelittin with time were investigated by incubating the oocytes in the presence of unlabelled valine (5 mM) for 40 h. During such a medium-length chase, the intracellular promelittin remains within the vesicle fraction

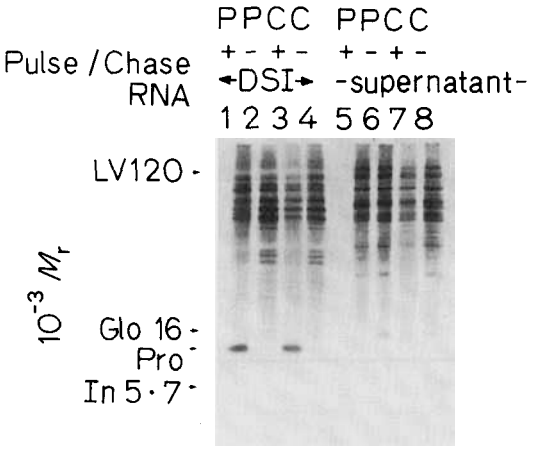

Fig. 4. *The subcellular distribution of promelittin made in oocytes under the direction of venom gland RNA.* Thus uninjected and RNA-injected frog cells were pulse labelled (P) for 40 h in [^{3}H]valine and oocytes were fractionated into cytosol (supernatant) detergent-solubilized vesicles (DSI) and yolk pellet. The remaining oocytes were incubated in medium for 4 h and, following the addition of valine (10 mM), the incubation was continued for a further 44 h. At the end of the chase (C), the oocytes were fractionated. Cell fractions were electrophoresed on 17.5% dodecyl sulphate/polyacrylamide gels. The positions of marker globin (Glo) and insulin (In) and lipovitellin (LV) are shown, as is that calculated for insect promelittin (Pro)

(see Fig. 4 and Table 1). There is also no qualitative and little quantitative change in the subpopulation of oocyte proteins that are sequestered: in this particular experiment the vesicle contents represented 17% of labelled oocyte protein at the end of the pulse labelling and 14% at the end of the 40-h chase period. Promelittin content, expressed as a percentage of total radioactive vesicle proteins, had risen slightly during the chase (experiment 1 of Table 1). A similar result was obtained in the second experiment shown in Table 1, which involved a much longer chase. The insect protein is, therefore, retained in this vesicle fraction somewhat more efficiently than the endogenous oocyte proteins.

Neither Melittin nor Promelittin Is Secreted Rapidly from RNA-Injected Oocytes

The specificity and requirements of the secretory system of the oocyte can be studied by the microinjection of heterologous messenger RNAs. The export of several foreign secretory proteins has been examined [7] and all were shown to appear in the incubation medium within 24 h, usually in amounts that can be detected by autoradiography on dodecyl sulphate gels. In contrast, we find that even after three days of incubation in the presence of radioactive valine, promelittin was not detectable in the medium, under conditions where radioactive proteins exported by oocytes and follicle cells were present in abundance

Table 1. *The relative amounts of promelittin and endogenous proteins within the oocyte vesicle fraction during a pulse-chase experiment*
Oocytes were injected with venom gland RNA and incubated with [^{3}H]valine; subcellular fractionation was performed after the initial labelling period (24 h for experiment 1 and 96 h for experiment 2) and following a 40-h or 144-h chase (experiments 1 and 2 respectively). Control oocytes were treated similarly. The amount of promelittin was measured by densitometery of gel fluorograms

Oocyte vesicle fraction	Promelittin content experiment 1		experiment 2	
	pulse	chase	pulse	chase
	%			
RNA-injected oocytes	7.0	10.6	21.4	27.4
Control oocytes	0	0	0	0

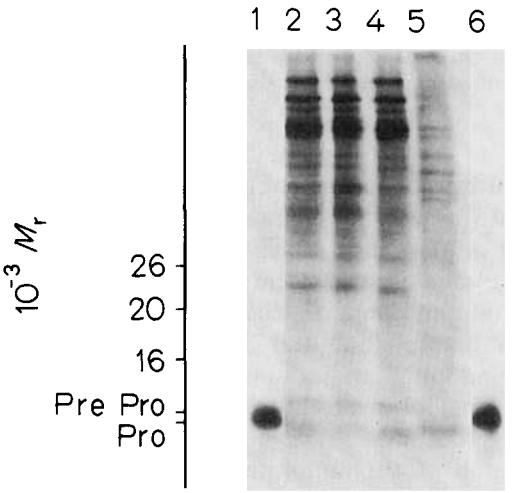

Fig. 5. *Proteins exported by oocytes injected with venom gland RNA.* Folliculated oocytes were injected with honey-bee RNA, and after an overnight incubation to permit healing of the micropipette wound, selected cells were cultured in [^{3}H]valine for three days. Uninjected control oocytes were labelled for the same length of time. The medium surrounding the amphibian cells was collected and analysed on a 17.5% dodecyl sulphate/polyacrylamide gel. Thus tracks 3 and 4 show medium from RNA injected oocytes, whilst that from controls is shown in track 2. Radioactive vesicle proteins, including labelled promelittin (Pro), were prepared from oocytes incubated for three days in [^{3}H]valine and were run as markers (track 5). [^{35}S]Methionine-containing prepromelittin (Pre-Pro) made in the wheat germ cell-free system was also run as a marker (tracks 1 and 6)

(see Fig. 5). In similar experiments with [^{3}H]proline, [^{3}H]leucine and [^{3}H]glycine, using both short and long labelling periods, secretion of promelittin could not be detected. However, on our gel system the protoxin usually runs as a diffuse doublet and thus as an assay gel autoradiography is rather insensitive. Consequently we checked for the presence of promelittin in the medium by the method used for isolating radioactive promelittin from venom glands. After

Table 2. *The amount of promelittin, melittin and acid-insoluble radioactivity in oocytes and their surrounding medium after labelling with valine, leucine and proline for various times*
Promelittin and melittin were partially purified and separated by butanol/water extraction. Specific fragments were prepared by enzymic digestion, purified by electrophoresis and their content of radioactive amino acid was measured (Materials and Methods). The amount of radioactive promelittin or melittin represented by a specific fragment was calculated from the known amino acid compositions of the intact proteins

Radioactive amino-acid and time of incubation	Radioactive promelittin in oocytes	Radioactive promelittin in medium	Radioactive promelittin inside cell/ radioactive promelittin outside cell	Radioactive melittin in medium[a]	10^{-6} × Acid-insoluble radio-activity in oocytes	10^{-6} × Acid-insoluble radio-activity in medium
	counts/min			counts/min		
Valine 68 h	95000	2400	40	< 500	41	1.29
Valine 68 h	194000	3800	51	< 500	53	1.29
Leucine 68 h	460000	32000	14	< 500	75	6.3
Leucine 68 h	970000	46000	21	< 500	23	3.5
Proline 40 h	200000	< 800	> 250	< 2800[b]	–	–
Leucine 20 h	3000	< 100	> 30	–	–	–

[a] Melittin could not be detected within oocytes [16].
[b] Purification of melittin by electrophoresis at pH 3.5 was omitted and the figure represents an upper limit for the melittin content of medium.

butanol extraction, paper chromatography and subsequent proteolytic digestion of the promelittin-containing fractions, small amounts of fragments characteristic of promelittin could be detected in the incubation medium. In the experiments shown in Table 2, between 0.4% and 7% of the total promelittin was found in the media. In no instance was there any enrichment for promelittin amongst secreted proteins; promelittin always represented a greater proportion of the radioactive proteins within than without the oocyte.

After extraction of the media with butan-1-ol we also checked for melittin formation. The butanol layers were therefore dried, digested with pepsin and then analysed for the presence of specific melittin fragments. No significant amounts above the low background, established by analysis of media surrounding uninjected oocytes, could be detected. Thus melittin constitutes less than 0.05% of the exported proteins. There is several hundred times as much promelittin inside as there is melittin outside the oocyte.

To assess the significance of the slow export of promelittin observed in these experiments, several factors have to be taken into account. It is conceivable that secretion of this insect polypeptide was, in fact, proceeding within the range of rates observed for other heterologous secretory proteins and that the secreted toxin or protoxin was unstable. Such a possibility was ruled out by measuring the relative amount of radioactive promelittin retained within the oocyte after incubation in the presence of excess unlabelled amino acid. As shown in Fig. 6 and Table 1, even after a six-day chase there is no loss of sequestered promelittin relative to sequestered oocyte proteins. Thus Fig. 6 shows a

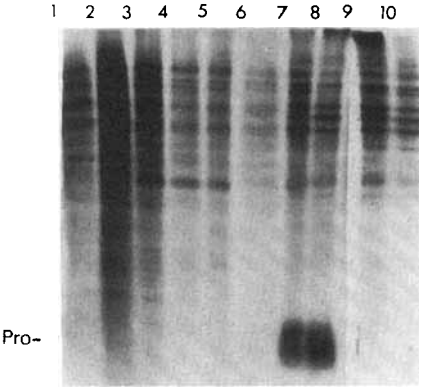

Fig. 6. *The long-term retention within a vesicle fraction of promelittin synthesized in oocytes.* Oocytes with and without added venom gland RNA were incubated for 96 h in [^{3}H]valine, before being transferred to unlabelled medium to which valine (10 mM) was added 4 h later. During the subsequent chase the incubation medium was changed at daily intervals. Gel analysis of the medium after the following incubation times is shown in tracks 1–6. Track 1 continuous labelling, 4-day period; track 2, chase 0–24 h; track 3, chase 24–48 h; track 4, chase 48–72 h; track 5, 72–96 h; and track 6, 96–120 h. Track 7 shows vesicle contents at the start of the chase, whilst track 8 depicts contents at the end of the 6 day chase, that is to say 10 days after [^{3}H]valine was added initially. Tracks 7 and 8 show vesicles from RNA-injected oocytes, whilst tracks 9 and 10, which also show vesicle contents at the beginning and end (respectively) of the chase, are from uninjected oocytes

diffuse band, absent from control oocytes, of the mobility expected of promelittin (these valine-labelled samples were rerun twice: the band in the promelittin region is reproducible, and on one gel the pulse-labelled material ran as a sharp band. Moreover the experiment was repeated using a different radioactive amino acid and a four-day chase; at the end

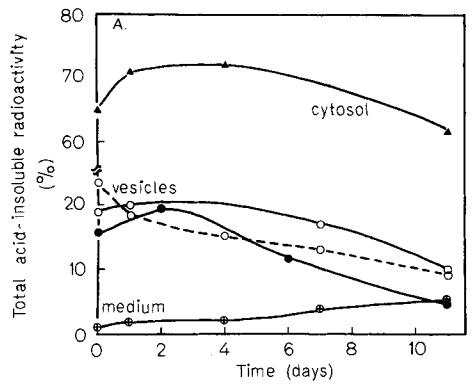

Fig. 7. *The changing distribution with time of radioactive protein within the oocyte vesicle fraction.* Oocytes were incubated in [^{35}S]methionine for 48 h (or [^{3}H]valine, for 24 h) and transferred to Barth X medium for 1 h; unlabelled amino acid was then added to yield a final concentration of 10 mM. The chase was continued for 12–13 days, the medium surrounding the oocytes being changed every 2–3 days. Unhealthy oocytes were discarded, as were dishes of oocytes showing signs of extensive leakage. At various times during the pulse-chase experiment groups of 10–20 oocytes were fractionated into cytosol, vesicle contents and yolk pellet. The surrounding theca and follicle cell layers were removed from some batches of oocytes by treatment with collagenase (2 mg/ml in 0.1 M phosphate buffer pH 7.4 for 90 min at 26 °C) before the initial labelling period. Thus the figure shows cytosol from folliculated oocytes (▲——▲), incubation medium surrounding folliculated oocytes (⊕——⊕), vesicle contents of folliculated (O---O), defolliculated (O——O) and venom gland RNA injected (●——●) oocytes. The oocytes injected with RNA came from a different frog and were labelled with [^{3}H]valine. Total acid-insoluble radioactivity within the system (oocytes plus medium) varies somewhat between batches of oocytes. However, no clear temporal trend is apparent, and nearly all values were within ± 30% of the levels of radioactivity recorded at the start of the chase

of the chase a diffuse band of proline-labelled promelittin was still visible in the vesicle fraction). Heptapeptide assay followed by sequential Edman degradation confirmed that vesicle fractions from pulse-labelled and pulse-chased RNA-injected oocytes contained valine-labelled promelittin, in similar amounts. The radioactive proteins of the oocyte vesicle fraction decline very slowly, during a chase (Fig. 7) yet promelittin is as or more stable than the endogenous vesicle proteins. An upper limit on the rate of exit of promelittin can be set by quantifying the rate of decline of the endogenous vesicle proteins: in a series of ten experiments using [^{3}H]valine and [^{35}S]methionine the percentage of the total radioactive protein (in oocytes and their medium) present in vesicles was measured as a function of time and yielded a half-life 13 ± 6 days. As shown in Fig. 7 the rate of decline of the endogenous vesicle contents is about the same in normal and venom-gland-RNA-injected oocytes, and the follicle cells surrounding the oocyte make little contribution either quantitatively or kinetically.

The small amounts of promelittin found in the incubation medium could actually result from leakage, as opposed to slow secretion, from the injected oocytes. Leakage is usually low, as shown by the similarity in gel profile of medium surrounding injected and uninjected oocytes (see Fig. 5). Nonetheless, at the level of detection covered by the assays for particular fragments leakage might be a prominent factor and, indeed, we argue that the highest inside/outside ratios recorded in Table 2, about 250:1, sets the upper limit for promelittin secretion and that lower ratios result from leakage.

DISCUSSION

The transfer of informational macromolecules characteristic of one cell type into the nucleus [25] or cytoplasm [1] of another can reveal the specificity of components involved in gene expression: transcription [26], translation [1], processing [5] and the subcellular or extracellular [7] destination of the processed protein can all be studied. The venom gland cell of the honey-bee produces melittin as the result of a complex series of cleavages and modifications. The *Xenopus* oocyte, the precursor of the totipotent egg cell, also has a functional secretory pathway [6,7]. Thus the specificity of the post-translational machinery, including the export systems, can be investigated by combining messenger RNA from the specialized insect cells with cytoplasm from the frog oocyte. In general, the events that follow the introduction of heterologous mRNAs demonstrate the lack of species or cell-type specificity in the systems for translation, processing, vectorial transfer and secretion [5–7].

Oocytes synthesizing promelittin follow only some aspects of this general rule. Firstly, enzymes within frog as well as venom gland cells seem capable of removing the signal sequence from the insect protein. Although in the oocyte there is no direct evidence of synthesis of the pre region [9,27], the subcellular localization (Fig. 4) and sequence of the newly made promelittin is compatible with the formation and accurate removal of the putative signal sequence. Studies on immunoglobin light chains formed in oocytes [28] also imply but also do not prove that the frog enzyme cleaves the newly made mouse light chain at the correct amino acid residue.

Secondly, oocyte promelittin is found in the vesicle fraction and not in the cytosol. Thus the early events in the biosynthesis of heterologous secretory proteins, such as cleavage of the pre peptide and vectorial transfer of the polypeptide chain through the membrane of the endoplasmic reticulum, proceed normally.

Thirdly, we show that oocyte, like venom gland promelittin has a frayed amino terminus: at least two forms, differing in length by two amino acids, are present. The shorter species, which was identified by

sequencing chymotryptic fragments, cannot be an artefact of digestion, since chymotrypsin will not cleave a Pro-Glu peptide bond. Moreover, in one experiment we sequenced the N terminus of intact promelittin and also observed the species shorter by two residues. The origin of the shorter species is not clear. It is probably not the result of incorrect processing of the primary translation product by oocyte signal peptidase, since none of the known signal peptides terminate with a proline residue [29]. Moreover, a specific messenger RNA does not code for the smaller species, since translation of promelittin mRNA *in vitro* yields a single product of uniform length [9]. We favour the view that after formation of promelittin in oocytes partial processing by a dipeptidylaminopeptidase can occur. A dipeptidylaminopeptidase IV has been isolated from mammalian tissues and cleaves preferentially after proline residues [14,15]. An enzyme of this type, isolated from pig kidney membranes or from queen-bee venom glands, can cleave dipeptides from promelittin [15a]. A similar enzyme may be present in frog oocytes but we do not know why cleavage of even the first dipeptide is incomplete and sometimes cannot be detected at all. Time does not seem to be a primary factor in this conversion, since in two experiments where oocytes were incubated for several days, the extent of processing had not increased significantly. Promelittin shorter by two residues can also be detected in queen-bee venom glands (Kreil, unpublished experiments) and there is good evidence that in this tissue the conversion of the precursor to melittin proceeds via stepwise cleavage of dipeptides. It should be emphasized that neither processing beyond the first or possible second depeptide nor melittin formation has been observed in frog oocytes.

The oocyte is also unable to form the amidated carboxyl terminus found in both promelittin and melittin from venom glands. Prepromelittin synthesized *in vitro* contains an extra carboxyl terminal residue and this glycine residue is also present in oocyte promelittin. Although promelittin is transferred across or into the endoplasmic reticulum of the oocyte, the post-translational reaction in which glycine is replaced by an amino group cannot be detected.

Finally, and again in contrast to the general rule that posttranslational events occurring in specialized cells can also take place in oocytes, we find that neither promelittin nor melittion is secreted rapidly by the oocyte. After a six-day chase much of the insect protein is retained, as the protoxin, within the vesicle fraction of the frog cell. Promelittin, but not melittin, can be detected in trace amounts in the medium surrounding the oocyte. The kinetics of secretion of promelittin are in striking contrast to the behaviour of all other heterologous secretory proteins so far tested in the oocyte system. Thus interferon, guinea-pig caseins, A, B and C and α-lactalbumin [7], chicken ovalbumin, conalbumin, ovomucoid and lysozyme, *Xenopus* albumin and vitellogenin, rat albumin and many other unidentified proteins from liver or oviduct can all be detected in substantial quantities in the medium from RNA injected oocytes (Lane and Colman, unpublished). The failure to secrete promelittin rapidly could be a consequence of combining an insect messenger with a frog cell. However, other insect proteins are secreted rapidly by oocytes, as shown by the injection of locust fat body RNA: four vitellin-like polypeptides, identified by immunoprecipitation and electrophoresis with locust egg vitellin are detected, within 12 h, in the incubation medium (Lane, Colman, Champion, James and Applebaum, unpublished).

The loss of promelittin from the vesicle fraction is even slower than that of the sequestered oocyte proteins. These endogenous vesicle proteins decline gradually over a period of several weeks and there is a concomitant rise in the radioactive protein in the medium. Two-dimensional gel analysis reveals that nearly all the species present in the incubation medium are also present in the vesicles (Mohun and Lane, unpublished), suggesting that some at least of the endogenous sequestered species are exported rather than degraded. Thus the injection of venom gland RNA into oocytes does not seem to inhibit the export of endogenous oocyte proteins; nor does it inhibit the secretion of heterologous proteins, as shown by the rapid export of chicken lysozyme from oocytes actively sequestering promelittin. The ratio of promelittin to endogenous vesicle proteins rises slightly during a long chase (Fig. 4 and 6, Table 1), whilst no relative enrichment of promelittin in the medium has ever been observed (Table 2). Moreover, the rate of accumulation of promelittin in the medium surrounding oocytes decreases with time, suggesting that the amounts of protoxin seen are the result of leakage. Thus there is no evidence that oocytes actively export the honey-bee polypeptide.

It is premature to draw a parallel between the failure to cleave and modify promelittin and its slow secretion. Although export has not been studied a similar situation may occur in frog cells injected with fish islet RNA. Thus, carp insulin cannot be detected, although proinsulin is seen in large amounts within oocytes [30]. We are investigating the possibility that promelittin is inserted into the membranes of oocyte vesicles as a transmembrane protein. Such an idea is not entirely without foundation, for thermodynamic calculations (as well as the observed properties of promelittin) suggest [31] that the insect protein might remain membrane associated even after signal sequence removal. Nonetheless, we stress that, for whatever reason, venom glands secrete promelittin rapidly [32] whilst oocytes secrete the insect protein slowly, if at all. Results obtained with

the frog cell system [7], and the demonstration that even bacteria can secrete chicken ovalbumin [33], suggest that some secretory mechanisms are common to all cells. In contrast, the failure of the oocyte either to destroy promelittin or secrete it rapidly suggests that not all secretory mechanisms function efficiently in all cells. Certain mechanisms may even be specific to certain cell types.

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REFERENCES

1. Lane, C. D., Marbaix, G. & Gurdon, J. B. (1971) *J. Mol. Biol. 61*, 73–91.
2. Woodland, H. R., Gurdon, J. B. & Lingrel, J. B. (1974) *Dev. Biol. 39*, 134–140.
3. Graessmann, A. & Graessmann, M. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 527–532.
4. Liu, C. P., Slate, D. L., Gravel, R. & Ruddle, F. H. (1979) *Proc. Natl Acad. Sci. USA, 76*, 4503–4506.
5. Asselbergs, F. A. M. (1979) *Mol. Biol. Rep. 5*, 199–208.
6. Zehavi-Willner, T. & Lane, C. D. (1977) *Cell, 11*, 683–693.
7. Colman, A. & Morser, J. (1979) *Cell, 17*, 517–526.
8. Lane, C. D., Shannon, S. & Craig, R. (1979) *Eur. J. Biochem. 101*, 485–495.
9. Suchanek, G., Kreil, G. & Hermodson, M. A. (1978) *Proc. Natl Acad. Sci. USA, 75*, 701–704.
10. Kreil, G., Suchanek, G., Kaschnitz, R. & Kindas-Mugge, I. (1978) *Proc. FEBS Meet. 47*, 79–88.
11. Habermann, E. & Jentsch, J. (1967) *Hoppe-Seyler's Z. Physiol. Chem. 348*, 37–50.
12. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol. 67*, 852–862.
13. Kaschnitz, R. & Kreil, G. (1978) *Biochem. Biophys. Res. Commun. 83*, 901–907.
14. Hopsu-Havu, V. K. & Glenner, G. G. (1966) *Histochemie, 3*, 197–201.
15. Kenny, A. J., Booth, A. G., George, S. G. & Ingram, J. (1976) *Biochem. J. 157*, 169–182.

15a. Kreil, G., Haiml, L. & Suchanek, G. (1980) *Eur. J. Biochem. 111*, 49–58.

16. Kindas-Mugge, I., Lane, C. D. & Kreil, G. (1974) *J. Mol. Biol. 87*, 451–462.
17. Ford, C. C. & Gurdon, J. E. (1977) *J. Embryol. Exp. Morphol. 37*, 203–209.
18. Berridge, M. V. & Lane, C. D. (1976) *Cell, 8*, 283–297.
19. Knowland, J. (1974) *Genetics, 78*, 383–394.
20. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.
21. Kreil, G. & Bachmayer, H. (1971) *Eur. J. Biochem. 20*, 344–350.
22. Suchanek, G. & Kreil, G. (1977) *Proc. Natl Acad. Sci. USA, 64*, 975–978.
23. Eppig, J. J. & Dumont, J. W. (1972) *Dev. Biol. 28*, 531.
24. Griffith, O. W., Novogrodsky, A. & Meister, A. (1979) *Proc. Natl Acad. Sci. USA, 76*, 2249–2252.
25. Mertz, J. E. & Gurdon, J. B. (1977) *Proc. Natl Acad. Sci. USA, 74*, 1502–1506.
26. de Robertis, E. M. & Gurdon, J. B. (1979) *Sci. Am. 241*, 60–68.
27. Suchanek, G., Kindas-Mugge, I., Kreil, G. & Schreier, M. H. (1975) *Eur. J. Biochem. 60*, 309–315.
28. Jilka, R., Familletti, P. & Pestka, S. (1979) *Arch. Biochem. Biophys. 192*, 290–295.
29. Habener, J. F., Rosenblatt, M., Kemper, B., Kronenberg, H. M., Rich, A. & Potts, J. T. (1978) *Proc. Natl Acad. Sci. USA, 75*, 2626–2620.
30. Rapoport, T. A., Thiele, B. J., Behn, S., Marbaix, G., Cleuter, Y., Hubert, E. & Huez, G. (1978) *Eur. J. Biochem. 87*, 229–233.
31. Heijne, G., Von (1980) *Eur. J. Biochem. 103*, 431–438.
32. Bachmayer, H., Kreil, G. & Suchanek, G. (1972) *J. Insect. Physiol. 18*, 1515–1521.
33. Fraser, T. H. & Bruce, B. J. (1978) *Proc. Natl Acad. Sci. USA, 75*, 5936–5940.

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