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MELITTIN SYNTHESIS IN THE VENOM SYSTEM OF THE HONEY BEE (*APIS MELLIFERA* L.)

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M. D. Owen and L. A. Pfaff. Melittin synthesis in the venom system of the honey bee (*Apis mellifera* L). *Toxicon* **33,** 1181–1188, 1995.—The amount of melittin (measured by a direct haemolytic assay) contained in the venom system of single honey bees (*Apis mellifera*), of known age, increases from the time of eclosion to an age of about 4 weeks when about 500 μg of melittin is present. In older bees (5–6 weeks) the melittin level falls to about 250 μg. Measurements of the incorporation of [^{3}H]leucine (injected into the haemolymph) into melittin show that melittin synthesis is most active in bees aged between 1 and 2 weeks after eclosion. The melittin content of the venom system changes as the summer progresses. Melittin levels in a bee of any age greater than 1 week are lower in mid-August than in a bee of the same age in early June.

INTRODUCTION

The first realization that the complex chemical mixture making up the natural venom of the honey bee (reviewed by Banks and Shipolini, 1986) changed with the age of the individual insect studied came in 1972 when Bachmayer *et al.* showed that tritiated leucine (made available by feeding in a glucose solution) was incorporated into melittin and its precursor peptides (melittin contains four leucine residues among its 26 amino acids) at different rates in bees of different ages. These results, combined with the older and less specific studies of Kaiser and Michl (1958) and Autrum and Kneitz (1959), demand that the age of a worker bee be considered in any quantitative study of the chemistry of bee venom. Age-related changes in the amounts of individual venom components have since been measured in studies of histamine (Owen *et al.*, 1974), hyaluronidase (Owen, 1979), the catecholamines dopamine and noradrenaline (Owen and Bidges, 1982), 5-hydroxytryptamine (5-HT) (Owen and Sloley, 1988) and phospholipase A_2 (Owen *et al.*, 1990). Two patterns emerge from this series of studies. The amine components of the venom (histamine, 5-HT, dopamine and noradrenaline) and phospholipase A_2 activity are all present at only low levels in the first week to 10 days of a worker bee's life and then increase rapidly, reach a maxima and fall to lower levels in very old (more than 6 weeks) worker bees. Measurements of hyaluronidase show a different pattern with almost the same hyaluronidase activity in the venom system of worker bee pupae before eclosion as that measured in older bees.

Measurements of dopamine and noradrenaline in worker bees of known age (Owen and Bridges, 1982) suggested that, in addition to age-related changes, there might also be variation in the quantitative composition of bee venom at different times during the summer. This suggestion was supported when measurements of 5-HT (Owen and Sloley, 1988) showed that in early June a 10-day-old bee venom gland and reservoir might contain less than 10 ng of 5-HT, while in late July a bee from the same colony and of the same age had about 175 ng of 5-HT in its gland and reservoir, and in late August the 5-HT content had fallen to about 50 ng/gland and reservoir.

The data presented in the Bachmayer *et al.* (1972) study of melittin synthesis in bees of different ages show an initial low level of promelittin and melittin synthesis, a rapid increase to a peak at 8–10 days and a steady decline to a level slightly below that at eclosion at 25 days. The rate of conversion of promelittin to melittin is minimal in very young bees and increases with age to its highest measured level in 20-day-old bees (older bees were not tested). This suggests that the combined data for promelittin and melittin, presented by Bachmayer *et al.* (1972), include measurements of proportionately more promelittin in younger bees and a greater amount of melittin in older bees. While the pattern of increase, followed by a fall, in this measurement is repeated in all of our measurements of other venom components, the timing of the increase and decrease in melittin secretion shown by Bachmayer *et al.* (1972) does not fit with measurements of other venom components (references above). Possible explanations for such differences might be found in the different local conditions prevailing in Austria and in SW Ontario. The geographic positions of Austria (latitude 47° N) and SW Ontario (latitude 42° N) create seasonal differences in physical conditions. Ontario has a shorter summer season, during which daytime temperatures are significantly higher than those in Austria, although summer day lengths will be longer in Austria (both factors would have significant effect on worker foraging times). The flower species harvested for nectar and pollen are likely to differ, and even if closely related, will bloom at somewhat different times in the summer. It is also possible that Bachmayer's (1979) results are affected by unrecognized seasonal variations [suggested by Owen and Sloley's (1988) measurements of 5-HT].

In these experiments we have (1) measured melittin activity (using a haemolytic assay) as an indicator of actual melittin levels, (2) estimated the rate of melittin synthesis (through the amount of injected [^{3}H]leucine incorporated into melittin) in bees of known ages, and (3) examined melittin levels in bees of the same age on different dates spread through the summer.

MATERIALS AND METHODS

Insect and venom preparation

Bees used in these experiments were 'Italian' strain *Apis mellifera* and were maintained in Langstroth hives by the bee keeping practices normal for the area (Townsend and Burke, 1972). Bees of known age were obtained by marking newly emerged bees (from a frame of capped brood kept in an incubator at 34°C and about 30% relative humidity), returning them to the hive and collecting them as required (Smith, 1972; Owen and Bridges, 1976). Bees of known age were anaesthetized with nitrogen before venom glands and reservoirs were dissected under insect saline (Pringle's insect ringer) and transferred to 500 μl of insect saline in a microtube. The gland and reservoir were homogenized by sonication (cooled in ice through 3 cycles of 10 sec at full power followed by 10 sec off) and tissue debris was pelleted by centrifuging for 2 min at 12,500 ***g***. The supernatant was used as the 'venom extract' in measurements of melittin in the venom system.

Haemolytic assay of melittin

Washed sheep erythrocytes (blood samples were obtained from the same animal throughout the period of the experiments) were prepared each day. Two millilitres of sheep blood (in Alserver's solution) was centrifuged

(8 min at 650 *g* in a clinical centrifuge) and the red blood cell pellet washed three times by resuspension in isotonic saline (0.85%) and then centrifuged. An approximately 0.25% cell suspension (100 μl of red blood cells in 40 ml saline) was prepared as the red blood cell substrate for the haemolytic assay.

Each assay tube contained 1 ml of red blood cell suspension, a volume of venom extract giving between 10% and 100% red blood cell lysis and 0.85% saline to produce a total volume of 1.5 ml. (Appropriate venom extract quantities were determined in pilot experiments and varied between 500 μl containing 10 venom reservoirs for 0-day-old bees to fractions of a reservoir in older bees.) Tubes were capped and incubated at 37°C for 30 min before absorbance was read at 413 nm. The absorbance equivalent to 100% lysis was determined for each red blood cell substrate preparation by osmotically shocking an aliquot of the substrate. For each venom extract % lysis was plotted against the amount of venom system present and recorded as the amount of venom extract required to produce 50% lysis. These results were converted to estimates of the amount of melittin present in each venom system by comparison with a graph of the % lysis caused by measured amounts of commercially purified (Sigma Chemical Co.) melittin.

Leucine incorporation

In mid- to late August bees of known age were removed from the hive, anaesthetized with nitrogen, and 1 μl of [^{3}H]leucine solution (L-[4,5-^{3}H]leucine in aqueous solution, Amersham TRK.510, diluted in insect saline to a concentration calculated to yield 30,000 DPM/μl) injected, using an Agla micrometer syringe with a glass pipette needle, through the intersegmental membrane of the first and second abdominal sternites. Leucine-injected bees were placed in containers supplied with water and honey in a 32°C incubator. After 24 hr these bees were anaesthetized with nitrogen and the venom system was dissected out in a 1 mM leucine solution. The venom system (handled by the chitinous shaft of the sting) was moved through three rinses in 1 mM leucine to a final leucine rinse where the venom gland and reservoir were cut away from the hard parts and transferred to 20 μl of a solution containing 2 mg/μl melittin in 1 mM leucine in a microtube. The reservoir was punctured several times and the gland broken with a fine needle before being centrifuged (12,500 *g*) for 2 min to compress the gland and reservoir and squeeze their contents into the supernatant. (The aim of this method of venom extraction was to achieve maximum extraction of pure venom from the lumen of the gland and reservoir but to minimize the extraction of venom component precursors, particularly promelittin, that are part of the yield from homogenized venom glands and reservoirs.) The supernatant was remixed and 15 μl spotted on a NM 300 μm cellulose plate. The plate was run for 6 hr with a solvent mixture of butanol (90 ml):pyridine (60 ml:glacial acetic acid (18 ml):water (72 ml), dried, sprayed with ninhydrin, redried and sprayed again with nihydrin. (This chromatographic technique, which provides a rapid and simple separation of the major components of honey bee venom, was developed by the late W. B. Elliott. We appreciate Bill Elliot's assistance with this technique in the early phases of this study.)

Melittin and leucine spots on the plates were scraped into vials, 0.5 ml of 5% HCl was added and thoroughly vortexed and the mixture kept at 4°C overnight before the addition of 4.5 ml of scintillation cocktail (4 g Omnifluor in 660 ml toluene + 330 ml Triton-X) for counting. Recovery of [^{3}H]leucine standards spotted on the plate was 31 $\pm$ 4% of the DPM applied at the origin.

RESULTS

Melittin levels in bees of different ages

Measurements of melittin by its direct haemolytic action, on washed red blood cells, are summarized in Fig. 1. Melittin is at a low level in newly emerged bees and increases only a little in the first week of adult life. There is then a rapid increase in melittin level through the second and third weeks of adult life, followed by a continuing but slower increase until week 5 after eclosion. Six- and seven-week-old bees show lower melittin levels.

Leucine incorporation into melittin

Through most of the life span of a worker bee the amount of [^{3}H] incorporated into melittin from the [^{3}H]leucine precursor (calculated as DPM) is relatively low (Fig. 2). However, between 9 and 12 days after ecolosion there is a peak in the amount of labelled leucine incorporated into melittin. This peak is about eight times higher than the level of [^{3}H]leucine incorporation into melittin measured through the rest of the life span.

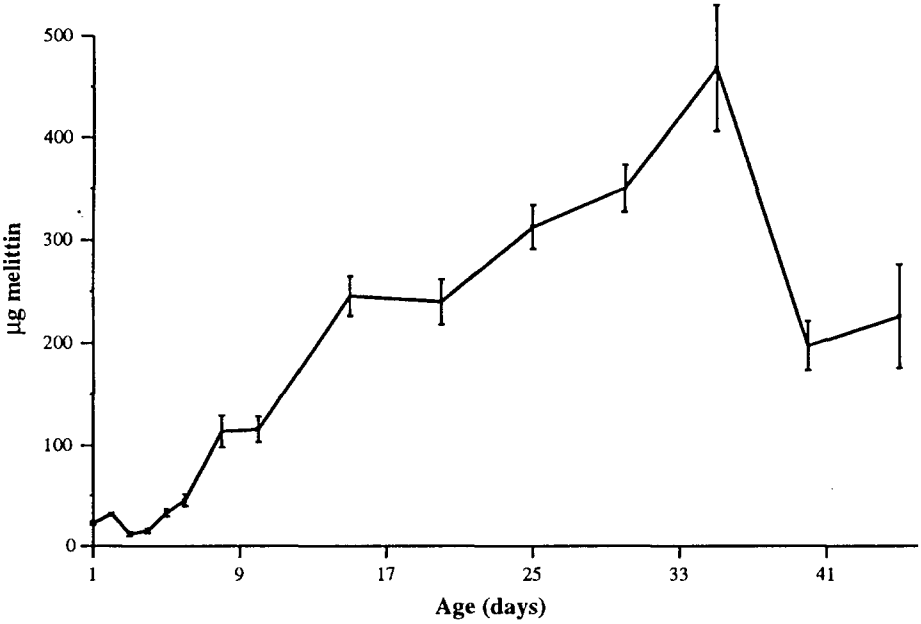

Fig. 1. The melittin content (measured by a haemolytic assay) of the venom system of honey bees aged 1–45 days after eclosion.
Each point is the mean of between 10 and 15 measurements made on two or three dates between 1 June and 19 August. Error bars show ±1 S.E.M.

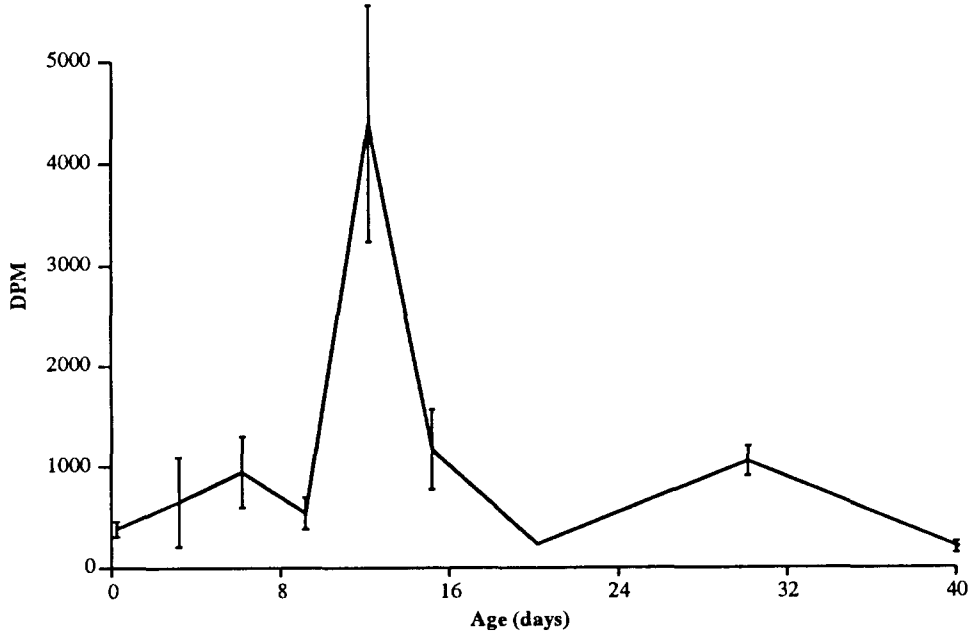

Fig. 2. The amount of [^{3}H]leucine (expressed as DPM) incorporated into melittin in bees aged 0 (day of eclosion)–40 days.
Each point is the mean of 5–7 determinations, made in mid–late August. Error bars show ±1 S.E.M.

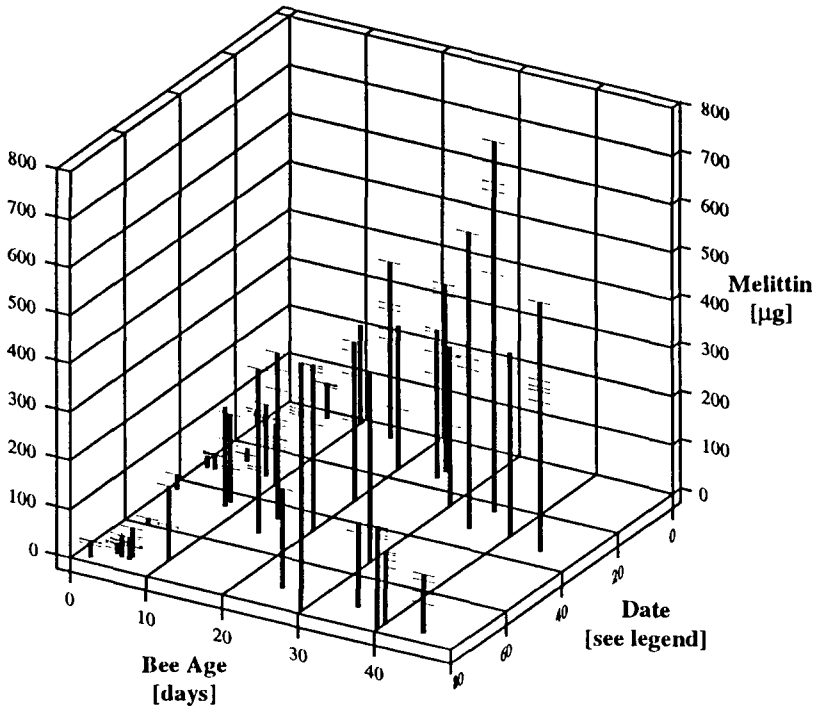

Fig. 3. Melittin content of the venom system, in bees of the same age, on different dates through the summer.

The height of each bar shows the maximum amount of melittin in a single bee of a given age on a given date. Fine lines across the bars show the melittin content of the venom system of the other bees of the same age, measured on the same date. Day 1 is 1 June; Day 31 is 1 July; Day 61 is 1 August; Day 80 is 19 August. ANOVA shows the change with the progress of the season to be significant ($P < 0.05$).

Seasonal variation in melittin levels in bees of different ages

Figure 3 shows the data for the amount of melittin, in the venom system of bees of known age, arranged by the date on which the measurement was made. This presentation of the data suggests that from 1 June (Day 1 in Fig. 3) to 19 August (Day 80 in Fig. 3) there is a reduction in the amount of melittin present in the venom system of bees at most of the ages measured (amounts in young bees may not show this fall). ANOVA (Systat) shows this reduction to be significant ($P < 0.05$).

DISCUSSION

Measurements of melittin (as indicated by haemolytic activity) in worker bee venom reservoirs show an age-related pattern (Fig. 1) that is more similar to that of histamine (Owen *et al.*, 1974) than other peptide bee venom components studied to date.

The haemolytic activity of melittin is synergistically increased by the presence of phospholipase A_2 (Vogt *et al.*, 1970). In natural bee venom the levels of phospholipase A_2 are maximally activated by the natural levels of melittin through most of the life of a worker bee (Owen *et al.*, 1990). Given the amounts of phospholipase A_2 and melittin in the venom it seems reasonable to conclude that the converse is also true. The commercial

melittin (Sigma Chemical Co., St Louis, U.S.A.) used as a standard is contaminated with phospholipase A (Sigma data); addition of more phospholipase A_2 does not cause a further increase in the haemolytic activity of the commercial melittin (unpublished results associated with Owen *et al.*, 1990). Consequently, our measurements of melittin by a haemolytic assay will overestimate the amount of melittin present but are directly comparable to studies using commercial melittin and are a reasonable indicator of the natural haemolytic activity of *Apis* venom.

A factor that most be considered in comparing our results with those of Bachmayer *et al.* (1972) is the different route by which labelled leucine was supplied, as a precursor, to the venom system. In the Bachmayer study the labelled precursor was added to the food; in our experiments leucine was injected into the haemolymph. Leucine, supplied in the food, is taken up only as a bee feeds and must cross the gut wall to the haemolymph before being available to the uptake mechanisms of the venom gland. We suggest that leucine supplied in the food would be available to the venom gland for longer periods of time, and at lower levels, than in our experiments. Injection of leucine into the haemolymph makes the labelled precursor directly and immediately available, as a single pulse, in the fluid circulating around the venom glands. It also produces an unnatural elevation in haemolymph leucine levels (although the low levels of labelled leucine in the melittin extract, at times away from the major peak of incorporation, suggest that this does not create a problem in the interpretation of the results).

The results shown in Fig. 2 suggest a much briefer time window in which maximal incorporation of labelled [^{3}H]leucine into melittin occurs than the rather broader peak in previous data (Fig. 1 in Bachmayer *et al.*, 1972). This difference may be explained by: (1) the nature of the extract of the venom system used (Bachmayer *et al.* used homogenates that included promelittin as well as melittin and labelled leucine would be a component of both compounds, our extraction aimed at isolating melittin from its precursor); (2) the route by which leucine is made available (Bachmayer *et al.* included the labelled precursor in food, in our experiments leucine was injected directly into the haemolymph); and perhaps (3) seasonal and nutritional differences in the conditions under which the bee colonies were maintained. A further difference between the earlier results (Bachmayer *et al.*, 1972) and this study was the period allowed between leucine feeding or injection and processing of the venom system. In the Bachmayer *et al.* experiments the conversion of promelittin to melittin appears to be still increasing 48 hr after removal from the source of the label. In pilot experiments we found maximal transfer of injected [^{3}H]leucine into melittin at about 24 hr after the injection of labelled precursor into the haemolymph.

An explanation of the apparent differences between the data in Fig. 1 (melittin level suggested by the haemolytic assay) and Fig. 2 (melittin synthesis) is provided in Fig. 4. The graph shows the cumulative [^{3}H] DPM (calculated from the data in Fig. 2) in melittin at all ages up to that sampled. This creates a crude model of the data that might have been obtained if labelled precursor had been continuously available for all melittin synthesis up to the age at which the venom system was examined. While we cannot relate DPM to actual melittin levels, the result of this modelling shows surprisingly good agreement with the shape of the curve generated from measurements of the haemolytic activity of melittin in bees of known age (Fig. 1 and replotted in Fig. 4). If labelled precursor had in fact been continuously available the leucine incorporation curve might be expected to both reach an overall higher level and to not plateau quite as markedly in older bees (cumulated counts from label incorporation are only at 5 day intervals through this part of the curve). Real melittin levels would be expected to be, on average, lower than the total amount

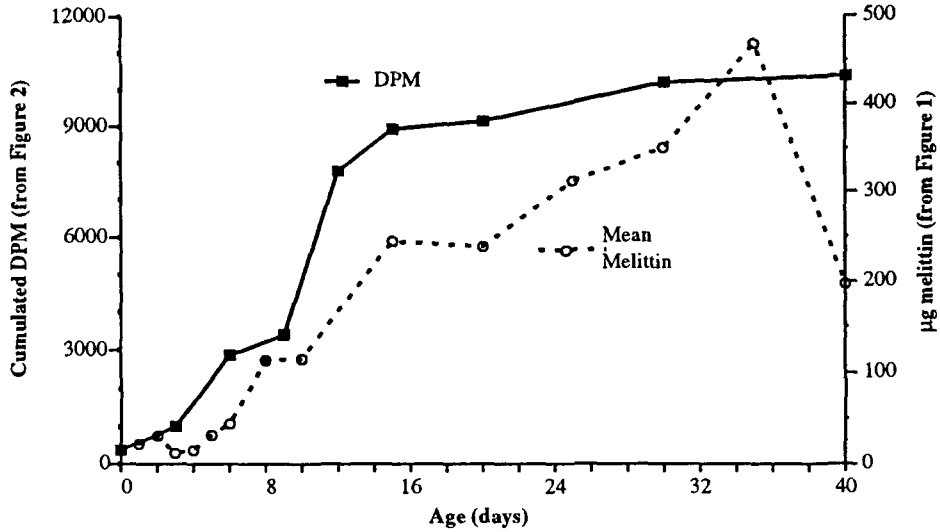

Fig. 4. Recalculated data from Fig. 2 superimposed on the data in Fig. 1. Each point on the DPM line shows the cumulation of the mean DPM from each of the samples up to the age indicated.

synthesized in a bee of given age as a result of venom use (the venom system is capable of resynthesizing venom; Owen, 1978) and venom leakage resulting from physical compression of the abdomen (Owen, 1979).

The earlier suggestions (Owen and Bridges, 1982; Owen and Sloley, 1988), based on measurements of dopamine, noradrenaline and 5-HT, that venom composition changes with seasonal factors, are supported by measurements of melittin, in the venom of bees of known age, at different times through the summer (Fig. 3). The venom system of a bee of any age greater than about a week contains less melittin in mid-August than would have been found in the venom system of a bee of the same age in early June. It is clear that the quantitative composition of honey bee venom is a dynamic system which is controlled, in large part, by the age of the bee and modified by external factors that are related to seasonal conditions.

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