

Crossed immunoelectrophoresis of major protein antigens during pupation of the leafcutting bee *Megachile rotundata*

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Summary — Antibodies to total homogenized prepupae of *Megachile rotundata* identified six major antigens in crossed immunoelectrophoresis. From the beginning of pupal differentiation until adult emergence, quantitative variation in six antigens was observed. Prepupal proteins from *Megachile relativa* demonstrated immunological identity with four of the six antigens identified in *Megachile rotundata* although one antigen differed in electrophoretic mobility.

Prepupal proteins were separated by isoelectric focusing in the first dimension and then run into a second-dimension, antibody-containing gel. Two antigens were correlated with polypeptides 4 ($M_r = 43,000$, $pI = 6.00$) and 1 ($M_r = 107,000$, $pI = 6.55$) of two-dimensional polyacrylamide gel electrophoresis analyses.

***Megachile rotundata* — development — protein fluctuation — crossed immunoelectrophoresis — voltinism**

Résumé — Immunoélectrophorèse croisée des principaux antigènes protéiques au cours de la nymphose de *Megachile rotundata*. Nous nous intéressons aux événements moléculaires qui sont à la base de l'émergence de *Megachile rotundata* dans les conditions naturelles. Nous avons utilisé l'immunoélectrophorèse croisée comme technique quantitative pour suivre les fluctuations des protéines nymphales.

Pendant 9 mois, nous avons injecté à des lapins les protéines totales homogénéisées des prénymphe de *M. rotundata*. Un pool de 817 ml d'antisérum a été récolté et utilisé pour obtenir une fraction d'immunoglobulines. Celle-ci a été utilisée pour précipiter les protéines au cours d'une immunoélectrophorèse croisée. Nous avons pu définir 6 pics d'immunoprécipitation et évalué leur variation quantitative au cours de la nymphose.

On n'a noté aucune fluctuation jusqu'au 10^e jour de l'incubation nymphale à 30 °C. Puis 5 des 6 pics ont diminué durant la fin de la période d'incubation. On a comparé les pics identifiés par l'immunoélectrophorèse croisée avec ceux obtenus précédemment par électrophorèse bidimensionnelle en gel de polyacrylamide. En séparant les protéines prénympheales par isoélectrofocalisation dans la 1^{re} dimension et en faisant migrer les protéines dans un gel de seconde dimension contenant les anticorps, on a pu associer 2 antigènes aux polypeptides 4 ($M_r = 43,000$

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$pI = 6,00$) et 1 ($M_r = 107.000$ $pI = 6,55$) obtenus en électrophorèse bidimensionnelle en gel de polyacrylamide.

Megachile rotundata — développement — fluctuation des protéines — immunoelectrophorèse croisée

Zusammenfassung — Kreuz-Immun-Elektrophorese der wichtigsten Protein-Antigene während der Verpuppung der Blattschneidebiene *Megachile rotundata*. Thema dieser Arbeit sind die molekularen Grundlagen des Auftauchens von *Megachile rotundata* während der Feldsaison. Zur quantitativen Erfassung der Verschiebungen in den pupalen Proteinen bedienten wir uns der Kreuz-Immun-Elektrophorese.

Völlig homogenisiertes Protein von *Megachile rotundata* — Präpuppen wurde Kaninchen neun Monate lang eingespritzt. Um eine ausreichende Immunglobulinfraktion zu erhalten, wurden 817 ml Antiserum gewonnen. Die Immunglobulinfraktion wurde verwendet, um in einer Kreuz-Immun-Elektrophorese die Proteine auszufällen. Sechs wohldefinierte Immunpräzipitat-Peaks wurden gefunden und während der Pupalentwicklung beobachtet.

Bis zum zehnten Tag der Inkubation der Puppen wurden bei 30°C keine Veränderungen beobachtet. Danach verringerten sich fünf der sechs Peaks über den Rest der Inkubationszeit. Ein Vergleich der Peaks aus der Kreuz-Immun-Elektrophorese mit einer vorherigen zweidimensionalen Polyacrylamid-Gel-Elektrophorese wurde durchgeführt. Bei der Auftrennung von präpupalen Proteinen durch isoelektrische Fokussierung in der ersten Dimension und anschließendes Laufenlassen der Proteine in der zweiten Dimension, Antikörper-haltiges Gel, zeigte sich ein Zusammenhang zweier Antigene mit Polypeptid 4 ($M_r = 43\ 000$, $pI = 6,00$) und Polypeptid 1 ($M_r = 107\ 000$, $pI = 6,55$) aus der zweidimensionalen Polyacrylamid-Gel-Elektrophorese.

Megachile rotundata, Verpuppung — Protein-Verschiebung — Kreuz-Immun-Elektrophorese

Introduction

The alfalfa leafcutting bee, *Megachile rotundata*, is an effective pollinator of alfalfa under a wide range of environmental conditions. A problem encountered in the management of this species is the variable field emergence of bees (Johansen *et al.*, 1973; Taséi and Masure, 1978; Taséi, 1982). This results in the production of a second generation of bees that is unable to reproduce before the onset of winter and generally is not effective in pollination. Furthermore, the second generation is believed to be a vector in the spread of chalkbrood (Vandenberg *et al.*, 1980). As a prerequisite to studying the physiological control of diapause development in this species we have investigated protein changes during pupation.

Total protein fluctuations during pupation in *M. rotundata* have previously been analyzed by the technique of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) utilizing isoelectric focusing in the first dimension and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis in the second dimension. Eleven polypeptides were observed to vary during development at 30 °C. The major polypeptide identified (apparent mol wt of 107,000 and isoelectric point of 6.55) decreased during development and was absent in newly emerged adults (Rank *et al.*, 1982). A major limitation of 2D-PAGE for physiological studies is the difficulty in quantitatively evaluating protein fluctuations. Crossed immunoelectrophoresis (CIE) provides a convenient quantitative evaluation of antigenic components (Laurell, 1966). This technique has been

used successfully in the analysis of mite allergens (Lind, 1980; Arlian *et al.*, 1985) and wasp venom (Einarsson *et al.*, 1985). CIE has not been used to evaluate protein fluctuations during insect pupation.

This study reports the identification of the major antigenic determinants in the prepupae of *M. rotundata*, the quantitative variation of these antigens during pupation, and an evaluation of proteins identified in common by 2D-PAGE and CIE analyses. The antigens of the related species *M. relativa* were also examined.

Materials and Methods

Antibody preparation

Fifty *M. rotundata* prepupae (stored at 5 °C c 5 mo) were homogenized in 40 ml of phosphate-buffered saline (pH 7.2), strained through wire mesh and sonicated for 4–5-s intervals. Samples at a protein concentration of 8 mg·ml⁻¹ were frozen in liquid nitrogen and stored at -80 °C. The prepupal homogenate was mixed 1:1 (v/v) with Freund's incomplete adjuvant and three rabbits were each injected intradermally with 500 µg of protein at 2-wk intervals. Eight days after the fourth injection serum was collected by bleeding through the marginal ear vein. Injections were continued at 3–4-wk intervals over a period of 9 mo and serum was collected every 8–10 d post-injection to produce an antiserum pool of 817 ml. An immunoglobulin fraction, in a final volume of 130 ml, was isolated from this pool according to the procedure of Harboe and Ingliid (1973). Plasmin was inactivated by the addition of aprotinin (200 KIU·ml⁻¹).

Incubation and sample analysis

Cells containing prepupae of *M. rotundata* and *M. relativa* were obtained from commercial hives (Rank and Goerzen, 1981) in the year following antibody production. After storage at 5 °C for 5 mo, prepupae to be used for electrophoretic analyses were incubated at 30 °C. Prepupae or developing pupae from

incubation periods of 0, 5, 10, 13, 18, and 21 d were ground in a teflon-glass homogenizer in phosphate-buffered saline (pH 7.2), mixed vigorously, strained through wire mesh, mixed vigorously, and sonicated (4 x 5 s) with a Fisher Ultrasonic probe set at the maximum power setting. The samples were quick frozen in liquid nitrogen and stored at -80 °C until used in electrophoretic analysis.

Electrophoresis

Samples prepared for CIE were adjusted to a protein concentration of 5 mg·ml⁻¹ in a buffer containing 1% (w/v) Triton X-100, 10 mM glycine, and 3.8 mM Tris, pH 8.7. The samples were sonicated (4 x 5 s) on ice with a Fisher Ultrasonic probe set at the maximum power setting, followed with centrifugation at 48,000 g for 60 min at 4 °C. The supernatant was removed and used within 2 h. Triton X-100, a neutral detergent, was included so that both membrane-bound and soluble proteins would be released.

Crossed immunoelectrophoresis was performed as previously described (Gerlach *et al.*, 1982). First-dimension electrophoresis was at 15 V·cm⁻¹ for 50 min in 1% (w/v) agarose gels containing 0.5% (w/v) Triton X-100. Second-dimension electrophoresis, on 7 x 10 cm glass plates, was at 2 V·cm⁻¹ for 18 h in 1% (w/v) agarose gels containing 200 µl of purified antibodies and 0.5% (w/v) Triton X-100. Immunoprecipitates were stained with Crocein scarlet—Coomassie brilliant blue (Crowle and Cline, 1977). Major immunoprecipitates were traced on Mylar, and peak area (Fig. 3) was determined by using a manual digitizer with a Hewlett-Packard model 9820A computer programmed to calculate the area of polygons. When CIE is performed according to Laurell (1966), the area of an antigen peak is directly proportional to the amount of the antigen present in the sample.

Samples for crossed immunoelectroforesis (CIF) were solubilized and subjected to first-dimension isoelectric focusing as previously described (Robertson *et al.*, 1980; Rank *et al.*, 1982). The gels were equilibrated in 2% (w/v) Triton X-100, 10 mM glycine, and 3.8 mM Tris, pH 8.7 for 45 min. The second-dimension electrophoresis, on 10 x 10 cm glass plates, was at 2 V·cm⁻¹ for 18 h in 1% (w/v) agarose gels containing 600 µl of purified antibodies and 0.5% (w/v) Triton X-100.

Results

Standard CIE pattern for prepupae

The antibody was produced with complete prepupae without any fractionation prior to intradermal injection. Similarly, a neutral detergent solubilization of total prepupal protein was employed which was capable of releasing both membrane-bound and soluble protein antigens (Gerlach *et al.*, 1982). Thus the CIE analysis represents the major antigens of the prepupae. As seen in Fig. 1, 6 well defined immunoprecipitates were observed. More than 20 independent analyses showed that the pattern of these 6 peaks was reproducible. Pronase digestion of the sample prior to CIE showed that the antigens were proteins (data not shown). The bilateral symmetry of the immunoprecipitates indicates that the proteins were not degraded prior to analysis. The low number of immunoprecipitates observed was consistent with previous 2D-PAGE analysis which identified only 8 major polypeptides in prepupae (Rank *et al.*, 1982).

Protein changes during pupation

The morphological development apparent after 5, 10, 13, and 18 d of incubation at 30 °C is given in Table I. No morphological differentiation was observed at d5.

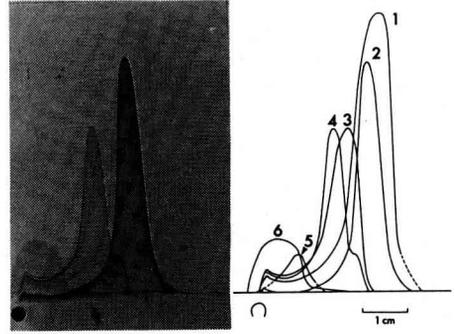


Fig. 1. CIE of *M. rotundata* prepupae. 20 μ l of Triton X-100 solubilized prepupal homogenate (100 μ g protein) was loaded in the application well (●). First dimension electrophoresis was performed at 15 V \cdot cm $^{-1}$ for 50 min and second dimension electrophoresis was at 2 V \cdot cm $^{-1}$ 18 h. The anode was to the right and top of the gel. The second dimension gel contained 200 μ l of purified antibodies to prepupal homogenates. A diagrammatic representation of the six reproducible immunoprecipitates is given on the right.

CIE analyses of d5 pupae also revealed no variation in the shapes or relative positions of the 6 immunoprecipitates (Fig. 2B). Similarly, an analysis of the area of the 6 immunoprecipitates showed minimal variation between d0 and d5 (Fig. 3). By d10, when morphological differentiation was observed, several of the immunoprecipitates had alterations in the peak area (Fig. 2C and Fig. 3). Several types of protein fluctuations were

Table I. Morphological characteristics of *M. rotundata* stages during development at 30 °C.

No. days	Morphological characteristics
0	Prepupa in diapause
5	No morphogenesis — appearance of prepupa as in diapause
10	Differentiation of head, thorax and abdomen
13	Melanization of pupal head and thorax; abdomen lightly striped with melanized bands
18	Melanization complete; wings undeveloped
Teneral adult	Newly emerged adult capable of flight

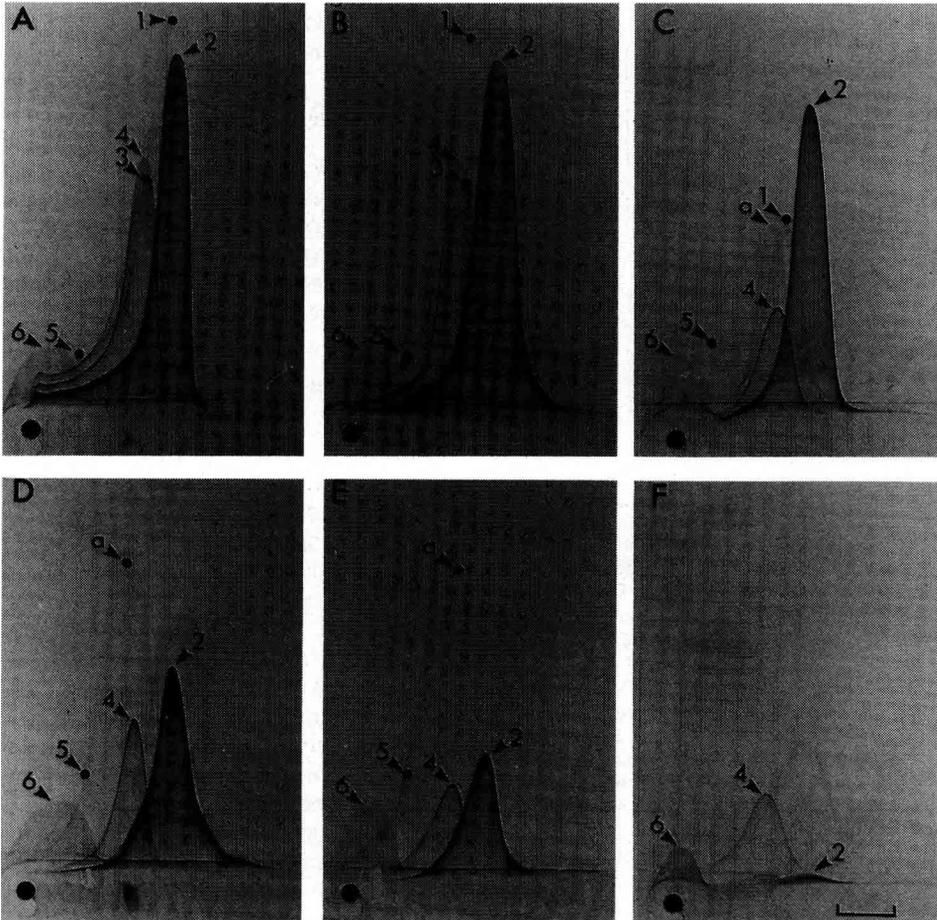


Fig. 2. CIE of *M. rotundata* during diapause development at 30 °C. A, prepupae; B, 5 days; C, 10 days; D, 13 days; E, 18 days; F, 21 days (teneral adults). Electrophoresis conditions were as in Fig. 1. Bar represents 1 cm.

observed during pupation. The area of peak 1 decreased from 7 to 2 cm², and peak 3 completely disappeared by day 10. By d13, peak 1 was no longer evident. Peaks 2 and 4 declined in area but did not disappear during pupation; peak 2 was virtually absent in teneral adults, whereas peak 4 declined to approximately one-third of the original value (3 cm² to 1 cm²).

Peak 6 did not change appreciably during development but appeared to decline slightly in adults. Peaks 5 and *a* increased during pupation but were absent in adults. The increase in the *a* immunoprecipitate is particularly striking as it was not evident in prepupal samples and subsequently increased to a maximum peak area of 7 cm² by d18.

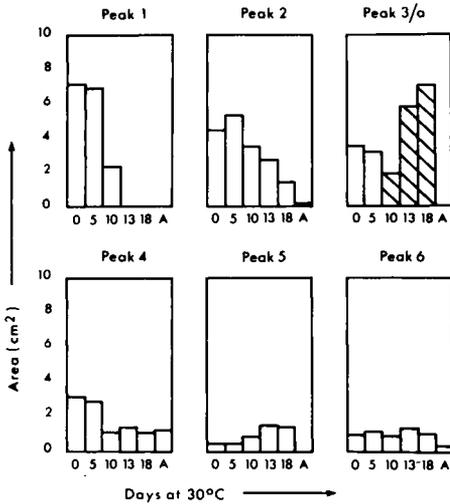


Fig. 3. Quantitative measurement of the immunoprecipitate peaks at 6 different stages of development as shown in Fig. 2. Visual inspection from 5 different analyses showed protein fluctuations to be highly reproducible. Quantification for a single set of analyses was performed as described in Materials and Methods. A = general adults that emerged on day 21. Note that areas at peak 3 and peak *a* (cross-hatched) have been combined in a single panel for ease of presentation since peak 3 was absent after day 5 and peak *a* was not observed until day 10.

Comparison of prepupal antigens of M. rotundata and M. relativa

Solubilized protein from *M. rotundata* and *M. relativa* prepupae were compared by CIE using the antibodies prepared against *M. rotundata* prepupae (Fig. 4). By mixing solubilized protein extracts from the two species it was possible to compare the antigens present in the *M. relativa* prepupae with those of *M. rotundata*. When the two samples were mixed, antigens 2, 3 and 6 gave single, symmetrical immunoprecipitates and therefore were antigens of similar electrophoretic mobility and complete

immunological identity. Antigen 4 of *M. rotundata* and antigen 4a of *M. relativa* produce a fused, bimodal immunoprecipitate with no spurs. This indicates that the two antigens have complete immunological identity but possess differing electrophoretic mobilities. Peaks 1 and 5 of *M. rotundata* are absent in *M. relativa*, suggesting that these antigens are either not present or else have little immunological similarity.

Comparison of 2D-PAGE and CIE analyses

Previous 2D-PAGE analyses had identified variations in 11 major polypeptides during pupation. These polypeptides ranged in apparent molecular weight (M_r) from 29 000 to 107 000 and apparent isoelectric point (pI) from 5.80 to 7.20 (Rank *et al.*, 1982). The hybrid technique of crossed immunoelectrofocusing (CIF) was used in an attempt to correlate these polypeptides with the antigens detected in the present CIE analyses. Proteins separated in a first-dimension, isoelectric focusing tube gel were run into a second-dimension gel containing antibodies to the prepupae of *M. rotundata* (Fig. 5). A comparative analysis of *M. rotundata* and *M. relativa* and the temporal variation in immunoprecipitate patterns was used to assist in protein identification.

M. rotundata preparations produced three tightly clustered, cathodal (*v*, *w*, and *x*) and one faint anodal (dotted line) immunoprecipitates (Fig. 5A). The pattern for *M. relativa* was similar except that only two cathodal immunoprecipitates (*y*, *z*) were identified (Fig. 5B). Protein denaturation resulting from the urea and SDS employed in solubilization limits the resolution possible with this technique. Nevertheless, tentative cross-identifications of proteins resolved by the 2D-PAGE and CIE analyses can be made.

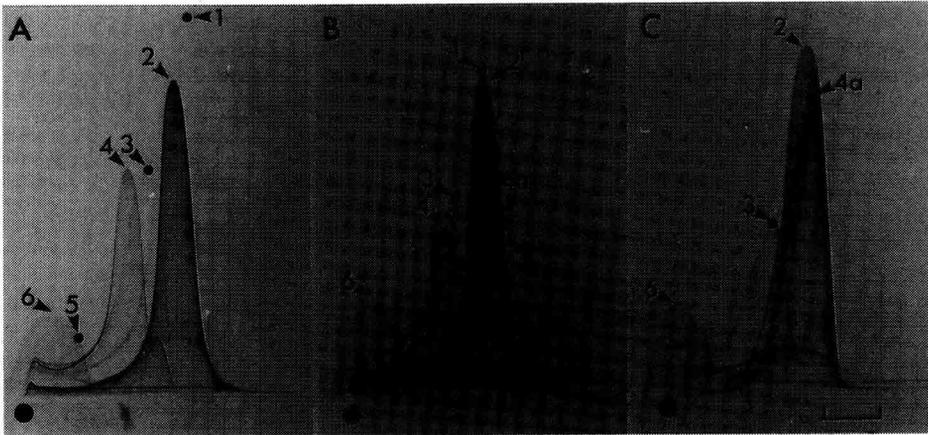


Fig. 4. Comparative CIE analysis of prepupae of *M. rotundata* and *M. relativa*. A, *M. rotundata* (100 µg protein); B, mixture of *M. rotundata* (50 µg protein) and *M. relativa* (50 µg protein); C, *M. relativa* (100 µg protein). Note that the antibodies employed were raised against prepupal homogenates of *M. rotundata*. Electrophoresis conditions were as in Fig. 1. Bar represents 1 cm.

The faint, anodal immunoprecipitate was common to both species (Fig. 5) and the electrophoretic mobility corresponds to peak 6 of the CIE analysis. Peak 6 area was constant during pupation (Figs. 2 and 3). Polypeptide 4 with an apparent M_r of 43,000 and apparent pI of 6.00 is the most likely equivalent component in the 2D-PAGE analysis since this is the only developmentally constant polypeptide with a low pI present in both species (Rank *et al.*, 1982; Table I). The positions of immunoprecipitates v (Fig. 5A) and y (Fig. 5B) coincide with the major component of the 2D-PAGE gel, polypeptide 1 ($M_r = 107,000$, $pI = 6.55$). The prominence of peak 2 in both species in the corresponding area of the CIE gel (Fig. 4) suggests that this immunoprecipitate contains polypeptide 1. This conclusion is supported by the observation that peak 2 and polypeptide 1 both decrease during development and are absent in general adults. The immunoprecipitates x , w , or z cannot be clearly correlated with any of

the remaining nine major polypeptides of the 2D-PAGE analysis. However, the absence of *M. rotundata* peak 5 (Fig. 4A) and immunoprecipitate x (Fig. 5A) in the corresponding *M. relativa* gels (Figs. 4C and 5B) indicates that these are the same antigenic component.

Discussion

Previous 2D-PAGE analysis identified 110 polypeptides in *M. relativa* and 104 polypeptides in *M. rotundata* prepupal homogenates (Rank *et al.*, 1982). In sharp contrast, the present CIE analysis identified only 6 and 4 antigens in prepupal homogenates of *M. rotundata* and *M. relativa*, respectively. However, of the 104 polypeptides identified by 2D-PAGE analysis of *M. rotundata*, only 11 polypeptides fluctuated in amount during pupation. Furthermore, an accurate and convenient quantitative evaluation of the

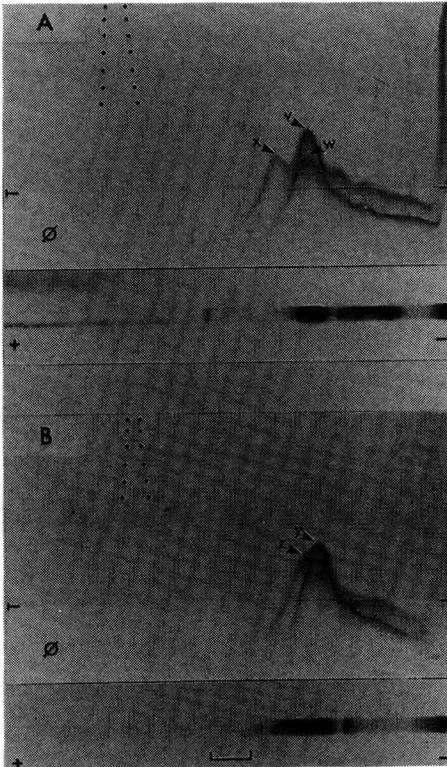


Fig. 5. Crossed immunoelectrofocusing of prepupae of *M. rotundata* and *M. relativa* to correlate components of 2D-PAGE and CIE analyses. Polypeptides separated in a first dimension isoelectric focusing polyacrylamide gel were run through an intermediate gel containing 1% (w/v) Triton X-100 (\emptyset) into a second dimension gel containing 0.5% (w/v) Triton X-100 and 600 μ l of antibodies to prepupae of *M. rotundata*. Second dimension electrophoresis was at 2 V \cdot cm $^{-1}$ for 18 h. The anode is to the left and top of the gels. The position of a weak immunoprecipitate not visible in the photographs has been indicated by a dotted line. A, *M. rotundata* prepupae; B, *M. relativa* prepupae. Bar represents 1 cm.

11 polypeptides was not possible. In the present analysis, the quantitative evaluation of fluctuations during pupation of all 6 antigens was possible. Thus the CIE analysis resolved fewer components

but, unlike 2D-PAGE, provided a convenient quantitative evaluation of resolved components. CIE analyses were rapid, inexpensive, and reproducible. The number of immunoprecipitates observed was consistent with the general observation of the previous 2D-PAGE analyses that polypeptide 1 accounted for greater than 25% of the prepupal protein, a situation analogous to that of the storage protein in *Calliphora* ssp. (Levenbrook and Bauer, 1980). In addition, the spot sizes of 4 additional polypeptides were greater than the other numerous minor polypeptides. Due to the relatively high concentration of these 5 polypeptides, the immunological response of rabbits injected with prepupal homogenates could be expected primarily to produce antibodies to only a few major proteins.

Solubilization of homogenates with a neutral detergent during immunization and CIE insured the analysis of both soluble and membrane-bound antigens. Thus the 7 immunoprecipitates defined by CIE represent the major protein antigens of the prepupae. The previous 2D-PAGE analysis monitored the total prepupal and pupal protein. The two analyses should therefore identify some of the same protein fluctuations. The polypeptides 1 and 4 of the 2D-PAGE analysis could be tentatively associated with the antigens identified in peaks 2 and 6, respectively, of the present CIE analysis. The peak 2 polypeptide was previously shown to have an apparent M_r of 107,000 and a pI of 6.55 (Rank *et al.*, 1982) and appears to be a major prepupal storage protein that is shunted into other proteins during pupation. The precise function and biochemical properties of the 6 antigens remains to be determined. *In situ* histochemistry, using intermediate gel techniques such as lectin binding and detergent charge shift will enable the further

characterization of these antigens. Such analyses, coupled with the quantitative evaluation of peak areas, should provide insight into the role of the major protein species during diapause development.

These results suggest that antigen 2 of the present analysis is an important marker for the field emergence of a second generation. The polypeptide carrying antigen 2 was shown by this analysis and by 2D-PAGE to dramatically decrease in content during pupation. Cells destined to become second-generation adults should be identifiable before morphological differentiation by a decrease in this important molecular marker. CIE will allow monitoring of the environmental induction of this trait at the molecular level and to compare the rate of diapause development between univoltine and multivoltine strains.

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