CHAPTER IV

RESULTS

1. Weight of *A. mellifera* in each developmental stage.

Each sample was weighed before an extraction. Samples were pooled at the 1st, 2nd, and 3rd stage because the samples was very light. The weight mean of samples in each developmental stage was recorded (Table 3).

Stage collection	Developmental stage	Mean of weight (mg)
1st (n=50)	The egg at 48 h	0.13 ± 0.05
2nd (n=30)	The first larva stage	0.48 ± 0.07
3rd (n=30)	The next 48 h larva stage	6.06 ± 3.72
4th (n=10)	The next 96 h larva stage	113.78 ± 14.65
5th (n=10)	The next 144 h larva stage	127.39 ± 8.08
6th (n=10)	The pre-pupa stage	123.16 ± 5.11
7th (n=10)	The white eye pupa stage	114.14 ± 4.52
8th (n=10)	The pink eye pupa stage	117.90 ± 4.00
9th (n=10)	The brown eye pre-adult stage	117.85 ± 0.66
10th (n=10)	The emerging stage	107.08 ± 2.32

Table 3. The weight mean of *A. mellifera* in each developmental stage was indicated.

2. Localization of AP activity of *A. mellifera* in each developmental stage

Whole mount of *A. mellifera* in each developmental stages was incubated in 350 μg/ml NBT and 175 μg/ ml BCIP (Change *et al*., 1993) for at least 3 h at 50°C or until the color changed from yellow to be purple. The sample would be incubated longer than 24 h before paraffin section. AP from whole mount of the 1st, 2nd, and the 3rd were detected in all tissue. In pupa at the 7th, 8th, 9th, and 10th stage, the incubation time requires longer because of hard cuticle. That made the AP activity less detected than another stages.

AP activity in egg (48 h) was found at tissue membrane such as chorion, vitelline membrane, etc. At the tip of egg, the activity was found at follicular epithelium and blastoderm (Figure 23 to 26).

All of larva at the 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} stages, the expression of AP was in integument tissue at cuticle, epidermis cell, and basement membrane (Figure 27 to 49). At a limb bud which will develop into segmentation, the activity was in epithelium cells (Figure 28-35, 41, and 42). The activity was always found at cell membrane, granules, and nuclear membrane (Figure 40, 42, and 45). In the gastovascular system, the activity was found in foregut, midgut, and hindgut. The activity in midgut was higher than in hindgut (Figure 36, 37, 38, and 43). Also, the lowest activity was in foregut. The highest activity showed at peritrophic membrane and vacuolar cell in midgut, especially in the 4^{th} stage (Figure 31, 36, 37, 43, and 44). The activity was also found in a tracheal system (Figure 38, 39, and 47). At the 2^{nd} stage, it was at proctodeum and anus, especially in ectodermal tissue. Proctodeum will develop to a mouthpart (Figure 44) and to an anus (Figure 27, 29, 30, and 32). At the 3^{rd}, 4^{th}, and 5^{th} stages, the activity was found at membrane, granule and around nucleus of fatty cells (Figure 39 and 40).

At the 6^{th} stage was prepupa development. Most of AP activity was at integument tissue such as in epidermis cell and cuticle. The activity was not found in other tissues because the cuticle at this stage will be formed more than in larva stages (Figure 50 to 52).

AP activity of the 7^{th}, 8^{th}, and 9^{th} were pupa stages. AP activity was hardly detected in internal tissue (Figure 40 to 69). Because they were formed hard cuticle. Also, they showed AP activity like other stages that were around integument tissue that was cuticle and epidermis cells (Figure 57 to 64, and 65). At head segment showed AP activity in antenna and mountpart (Figure 56). At all segment of legs (coxa, trochanter, femur, tibia, tarsus, and pretarsus) was found highly AP activity especially a join between segment (Figure 55, 59, and 69). An internal organ was shown activity in gut and free cells in gut (Figure 60 and 67), optic cell in compound eye (Figure 64 and 65), muscle cell in thorax (Figure 58 and 61), free fatty cells in tissue (Figure 62).

At the last stage was the emerging adult (10^{th}) (Figure 70). AP activity was the lowest detection in whole mouth because the integument was formed much hardly cuticle. When they were paraffin section were not found activity in internal tissue. But the AP activity can be detected when to be cut the integument. From section in this stage, AP activity were localized at all of integument, legs, sting, all part of gut, tracheal, and abdominal ganglia (Figure 71 to 74).

Figure 23 - 26. Localization of AP activity in egg (1^{st}).

a	—	position of cleavage cell
b	—	cell membrane of egg: vitelline membrane and chorion
c	—	blastoderm
d	—	yolk cell

Figure 27 - 31. Localization of AP activity in the first stage larva (2^{nd}).

A	—	anus
M	—	mouth
e	—	old cuticle
f	—	cuticle at proctodeum at mouthpart of larva
g	—	cuticle at anus
h	—	limb bud of larva
i	—	epithelium cell in limb bud
j	—	fatty body cell
k	—	free granule in fatty body cell
l	—	epithelium cell of gut
m	—	peritrophic membrane of gut
n	—	free AP in gut

Figure 32 - 37. Localization of AP activity at the next 48 h stage larva (3^{rd}).

o — new cuticle

p — epidermis

q — free granule in epithelium cell in gut

Figure 38 - 50. Localization of AP activity at the next 96 h stage larva (4^{th}) and next 144 h stage larva (5^{th}).

B — plasma membrane or cell membrane

G — gut

N — integument

V — epithelial cell

Tc — tracheae

★ — nucleus and nuclear membrane

x — basement membrane

y — free AP in cell

1 — foregut

2 — midgut

3 — hindgut

Figure 53- 74. Localization of AP activity at the next 192 h stage prepupa (6^{th}) and the next 240 h stage pupa (7^{th}), the next 288 h stage pupa (8^{th}), the next 336 h stage pupa (9^{th}), and the next 384 h stage emerging adult (10^{th}).

H — head part

T — thorax part

D — abdomen part

C — cornea lens

E — eye

J — matrix cell of cornea

P — pigment cell

W — reticula cell

F — antennae

K — mouthpart

L — legs

Q — optic lobe

R — malpighian tubule

S — glossae

Z — ganglia

8 — muscle cell

? — unidentify

Result of control in whole mount and tissue section of localization AP activity of *A. mellifera* in each developmental stage not be able to find purple to blue color in tissue. Data of control group not show.

Development stage	Localization of AP in tissue	Remark
Egg	- membrane of egg - cleavage cell - blastoderm - yolk cell	- vitelline membrane and chorion
Larva $2^{nd} - 5^{th}$ * high AP activity	- integument - alimentary canal - respiratory - other tissue	- cuticle, basement membrane, epidermis cell - epithelium cell of all gut, peritrophic membrane in mid gut, free AP in gut - epithelial lining at trachea - epithelium cell at limb buds: cell membrane, around nucleus, and free AP in cell - fatty body cell: membrane, around nucleus, and free AP in cell
Pupa – emerging adult $6^{th} - 10^{th}$	- overall integument - head - thorax - abdomen	- cuticle - compound eye: reticular cell, cornea lens mountpart: epithelial lining at glossae - antenna - muscle cell: membrane, around nucleus, and free AP in cell - epithelium cell of legs - alimentary canal: forgut, highly activity at midgut and hindgut - high activity at malpighian tubule - trachea - muscle at sting sheath

Table 4. All localizations of AP activity in tissue of *A. mellifera* in egg, larva, pupa, and emerging adult stages.

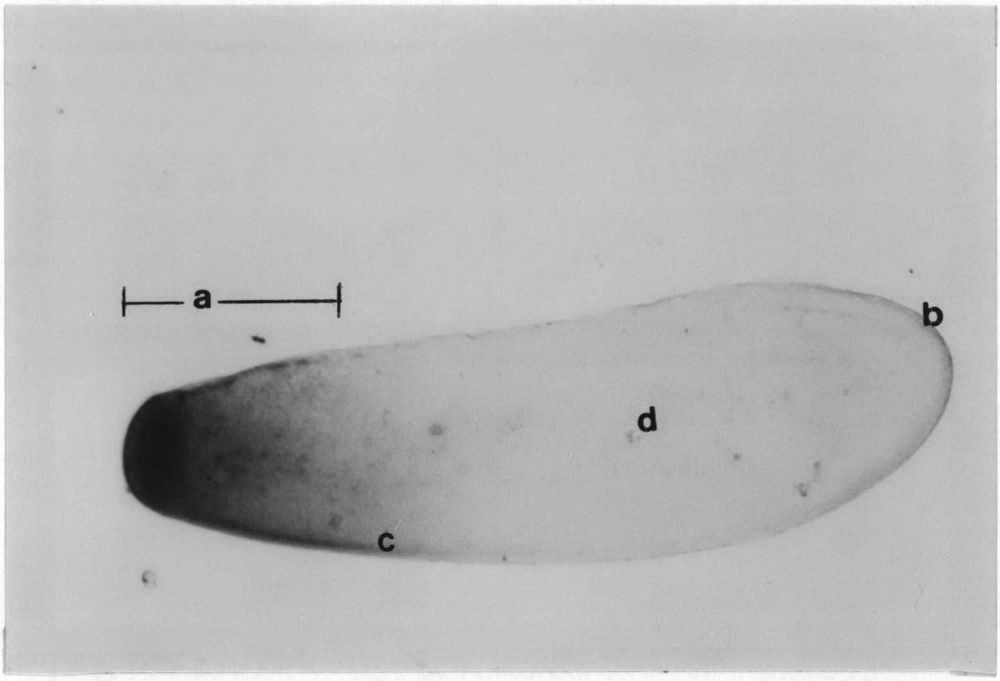


Figure 23. AP activity of the stage egg (1^{st}) was recorded with light microscope (LM) magnification objective lens ×4, and use Nomaski technique.

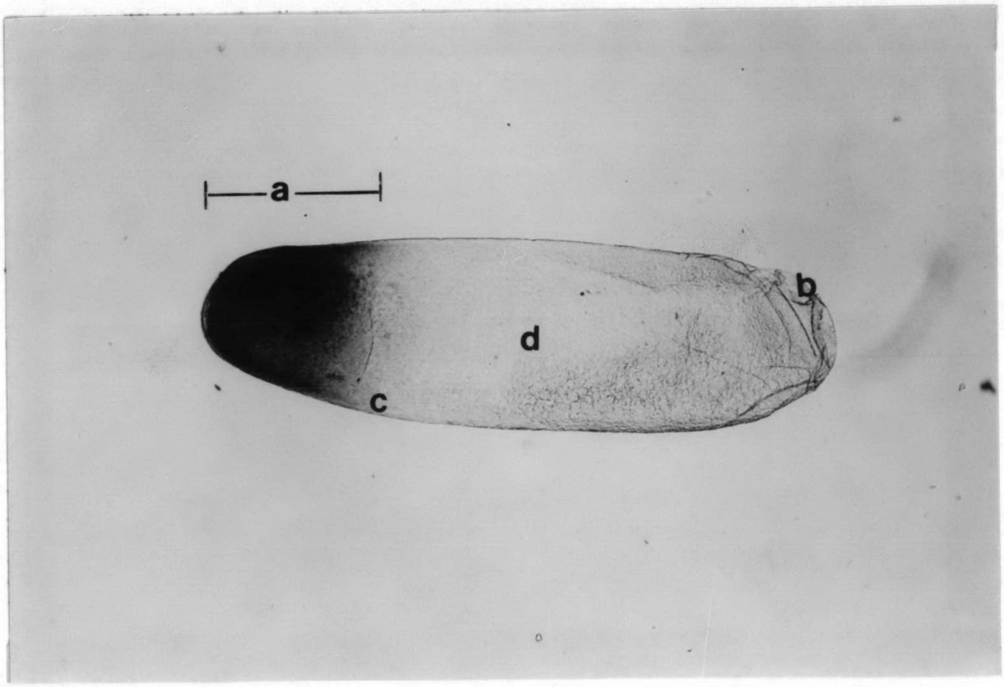


Figure 24. The whole mount at egg stage was recorded by LM magnification objective lens ×4.

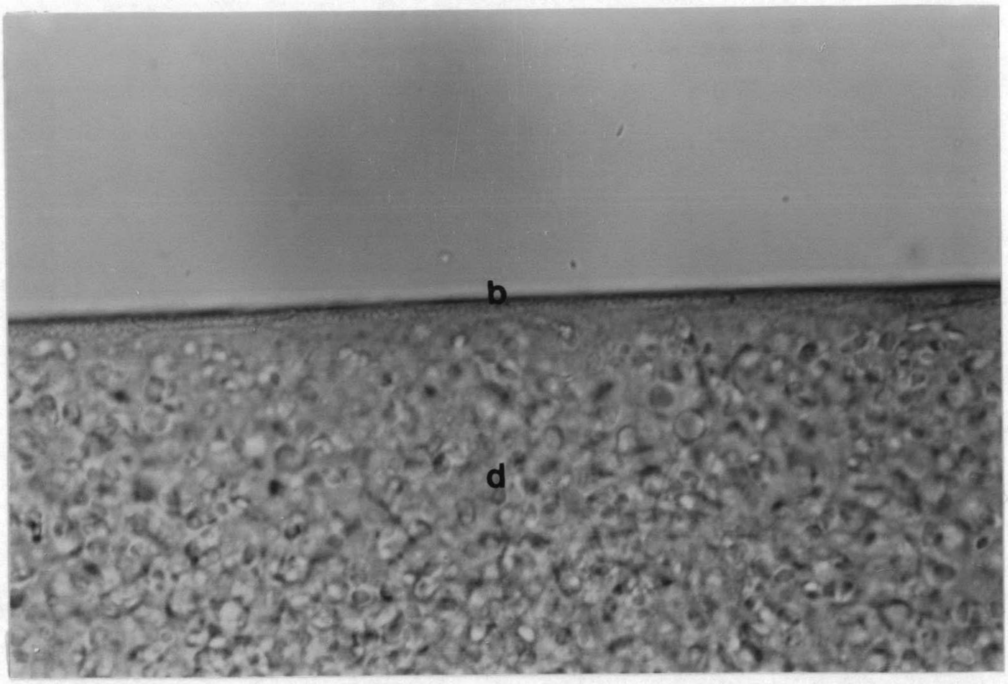


Figure 25. The membrane of egg stage was recorded by LM magnification objective lens ×40.

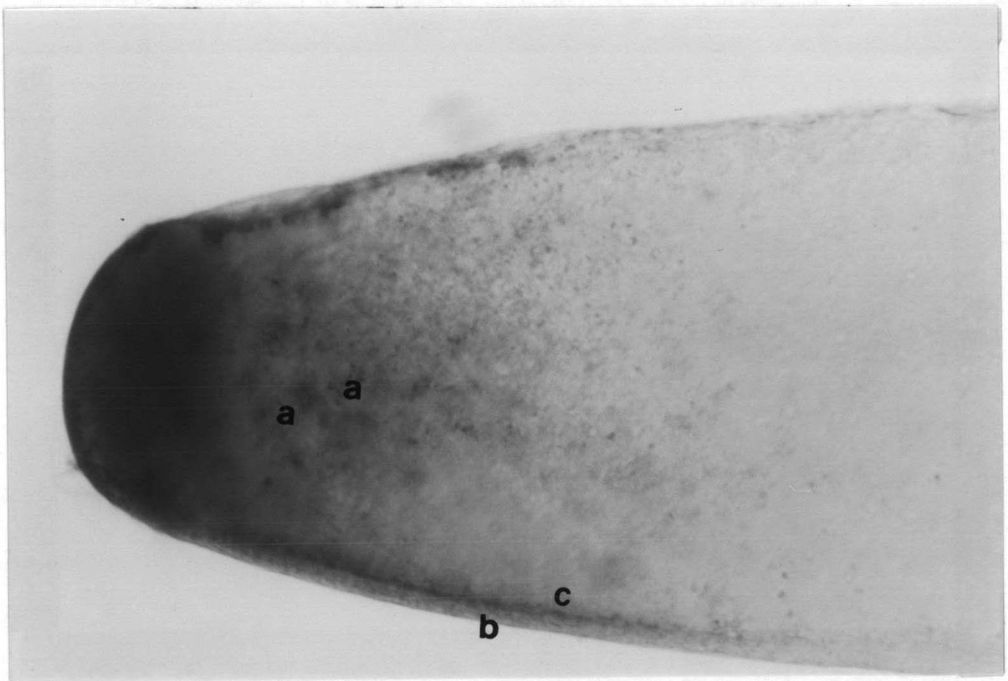


Figure 26. The whole mount at AP localization in egg stage was recorded by LM magnification objective lens ×10.

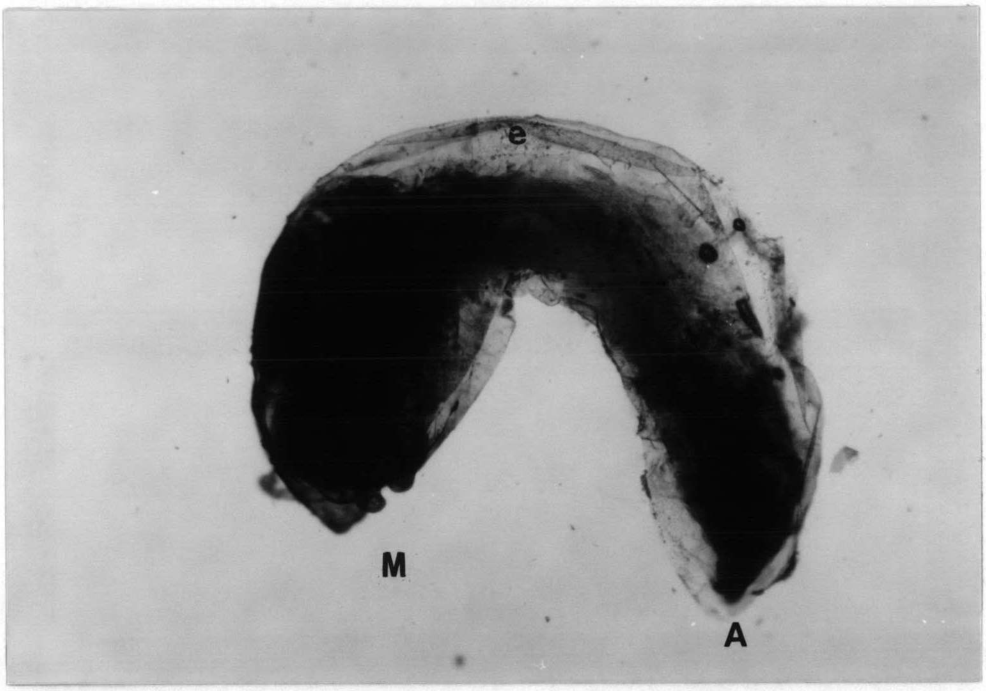


Figure 27. A whole mount of 2nd larva was photographed by LM magnification objective lens ×4.

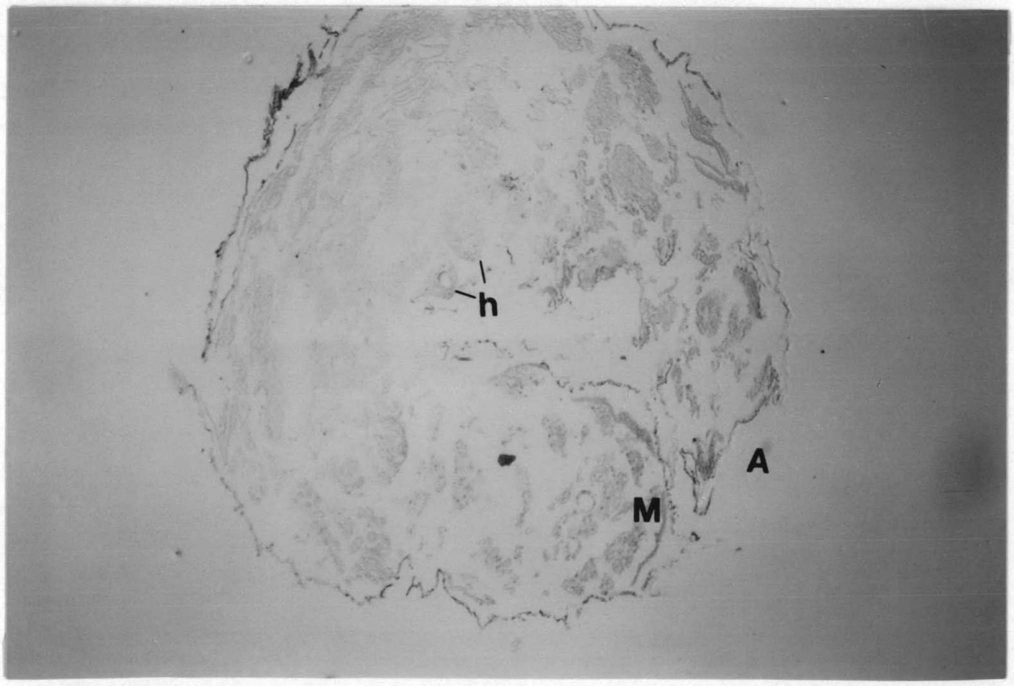


Figure 28. Section of 2nd larva was photographed by LM magnification objective lens ×4.

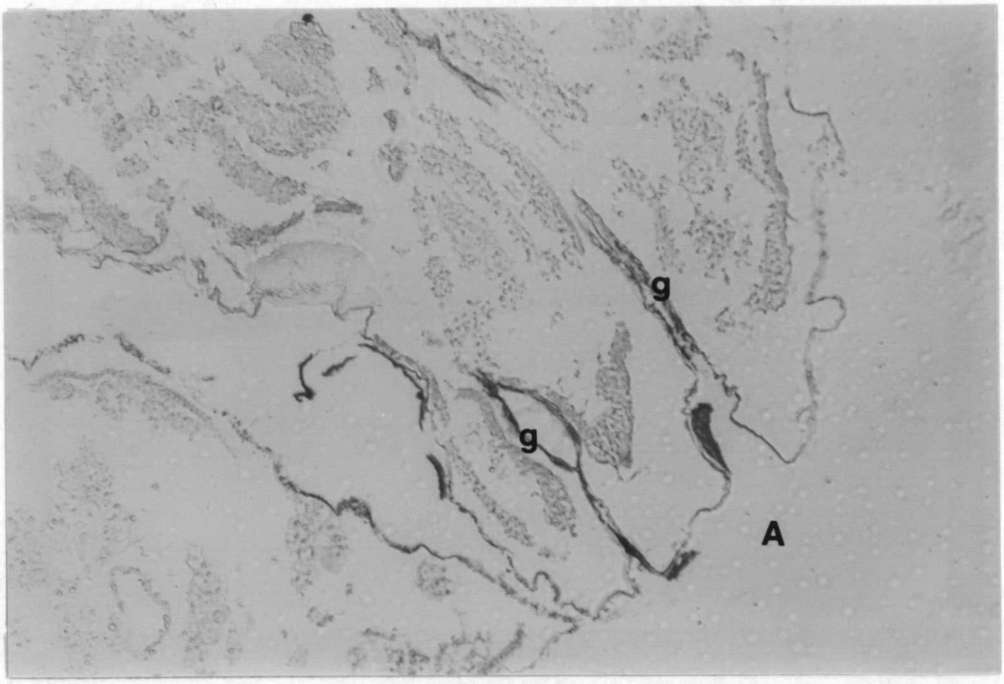


Figure 29. Section of 2nd larva was photographed by LM magnification objective lens ×10. That showed cuticle invagination at anus.

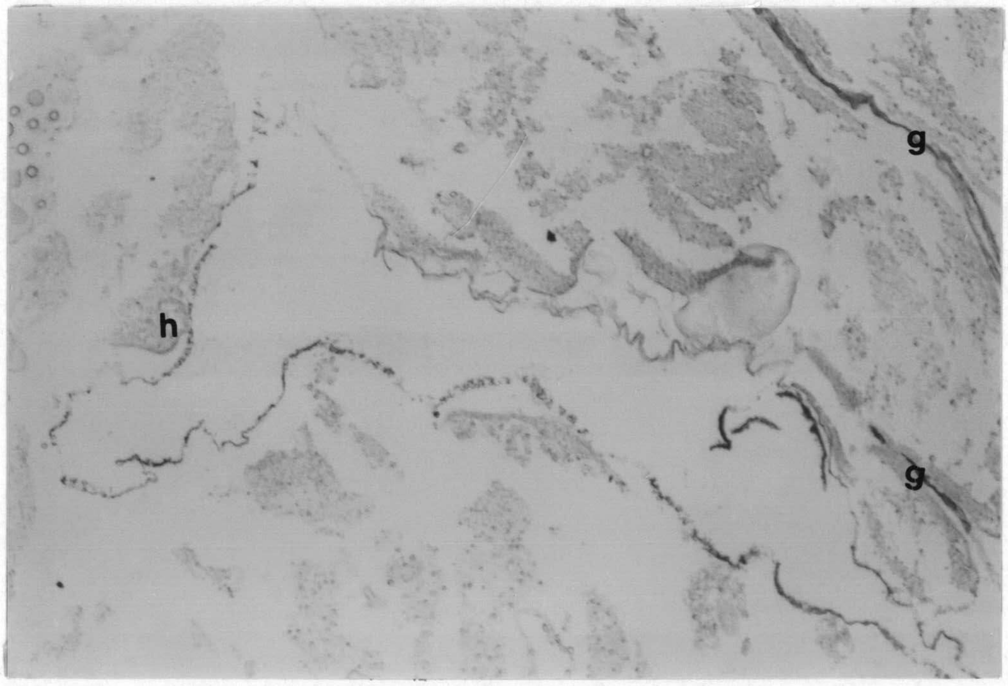


Figure 30. Section of 2nd larva was photographed by LM magnification objective lens ×10. That showed AP activity at limb bud and fatty body.

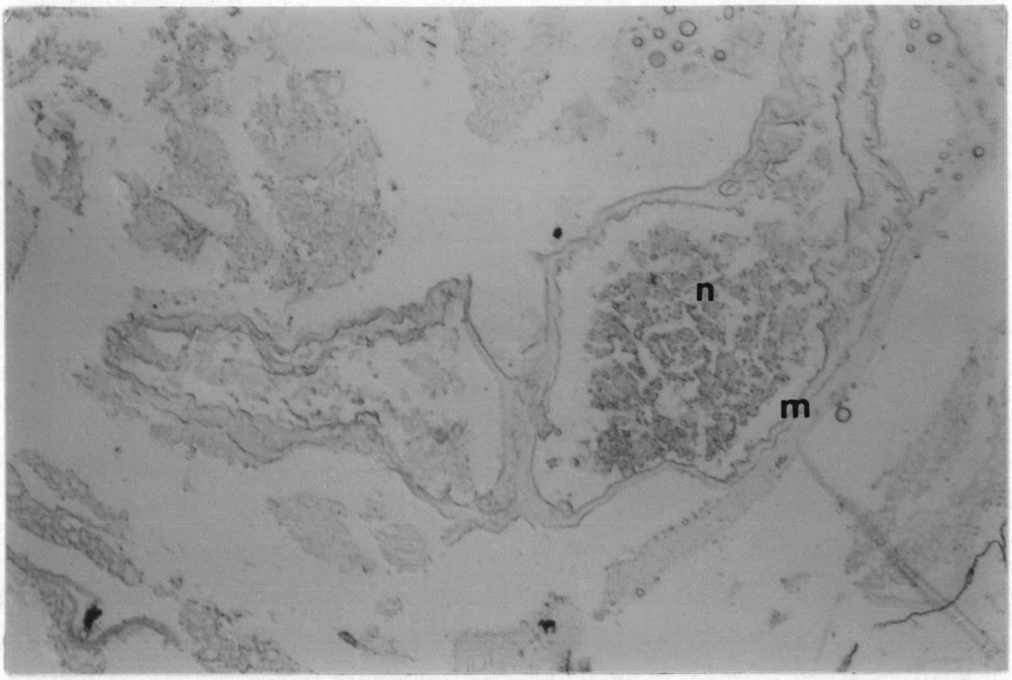


Figure 31. Section of 2[nd] larva was photographed by LM magnification objective lens ×10. That showed AP activity in gut, peritrophic membrane.

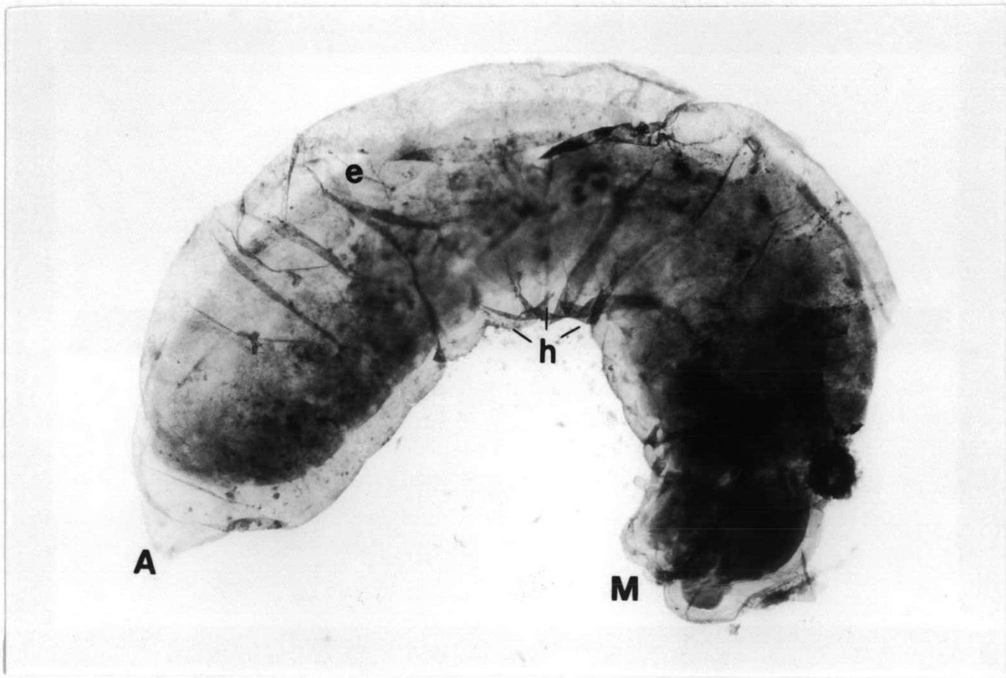


Figure 32. A whole mount of 3[rd] larva was photographed by LM magnification objective lens ×4.

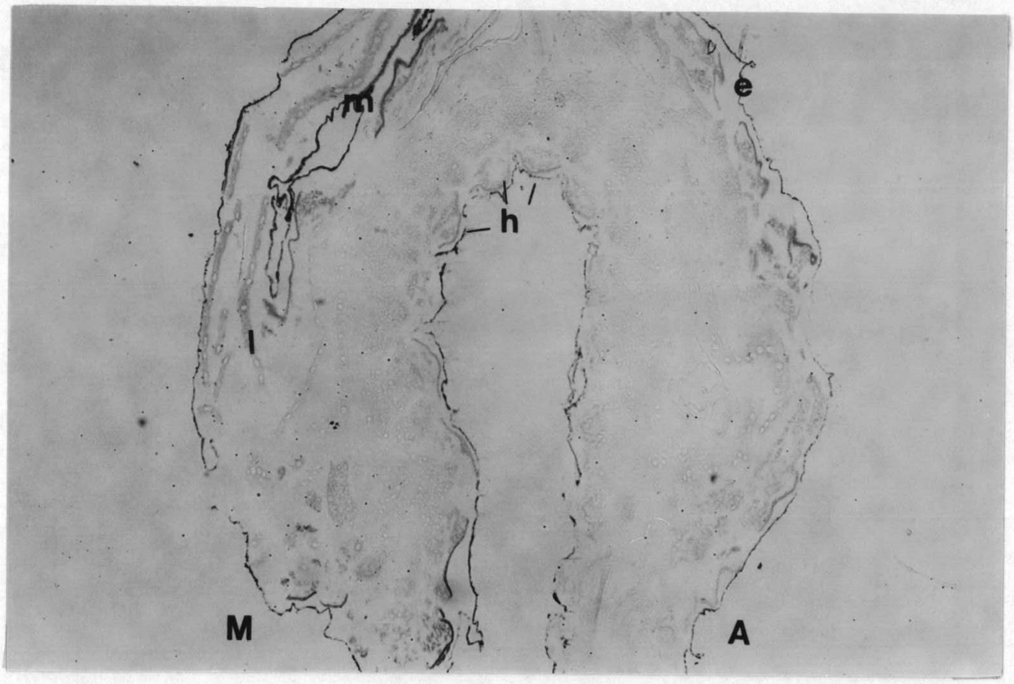


Figure 33. A section of 3[rd] larva was photographed by LM magnification objective lens ×4. That showed AP activity at integument, limb buds, at protodeum, and anus.

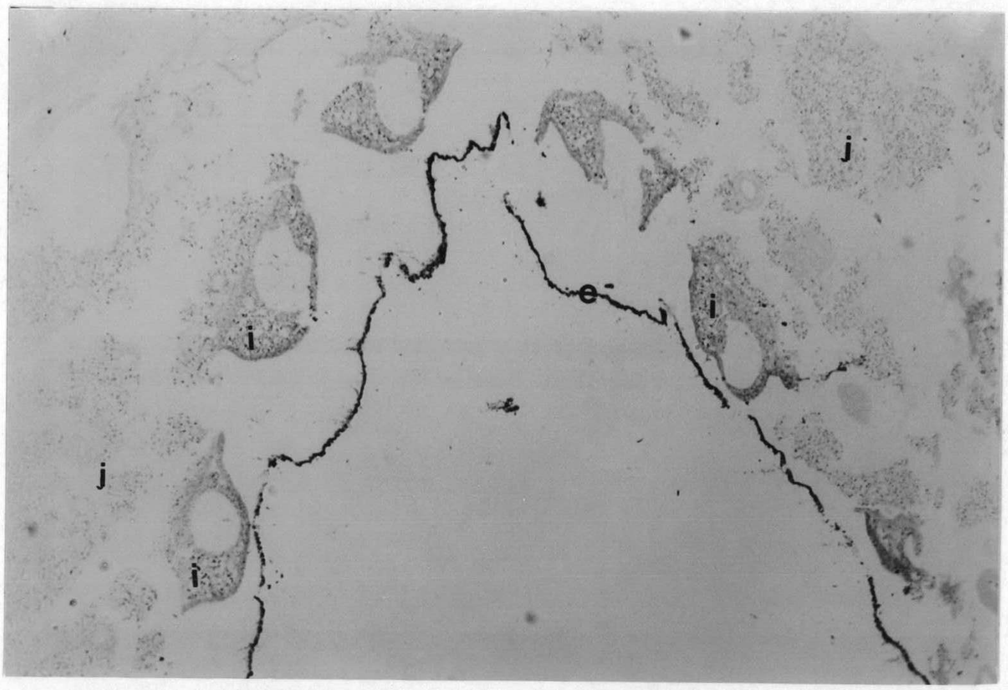


Figure 34. A section of 3[rd] larva was photographed by LM magnification objective lens ×10. That showed AP activity at epithelial cell at limb buds.

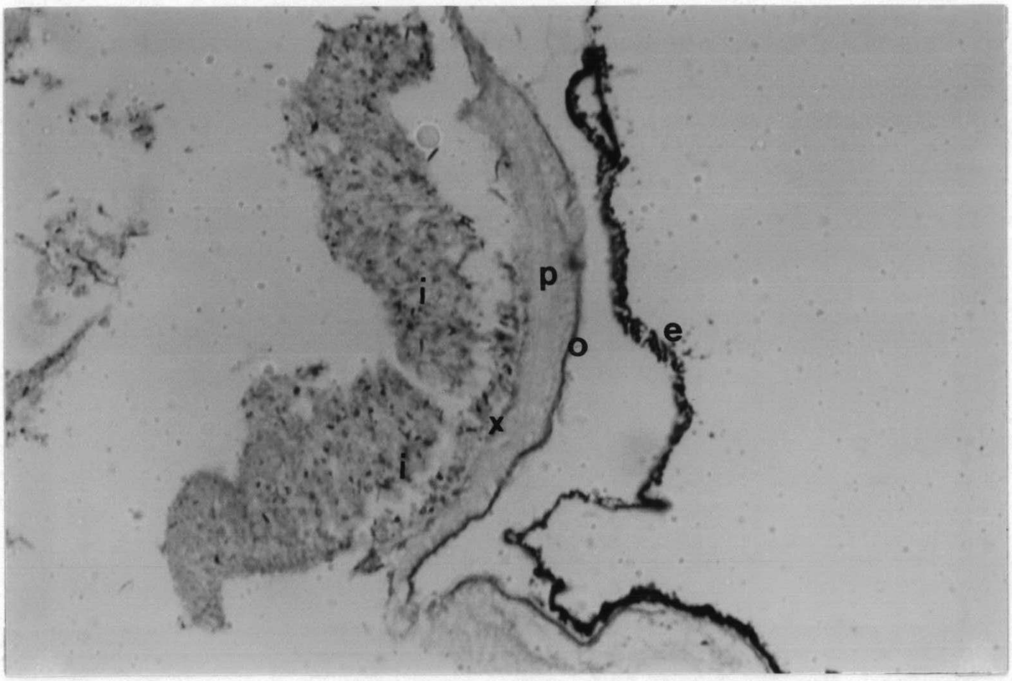


Figure 35. Section of 3rd larva was photographed by LM magnification objective lens ×40. That showed AP activity at limb buds.

Figure 36. Section of 3rd larva was photographed by LM magnification objective lens ×10. That showed AP activity in gut.

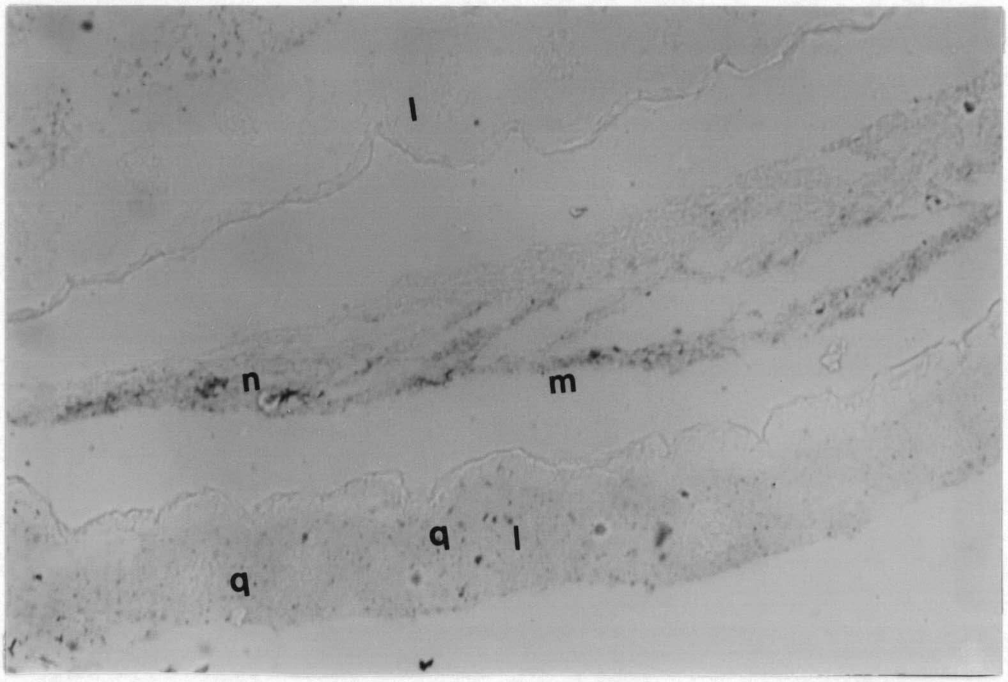


Figure 37. Section of 3rd larva was photographed by LM magnification objective lens ×40. That showed AP activity at epithelial cell of gut and free AP.

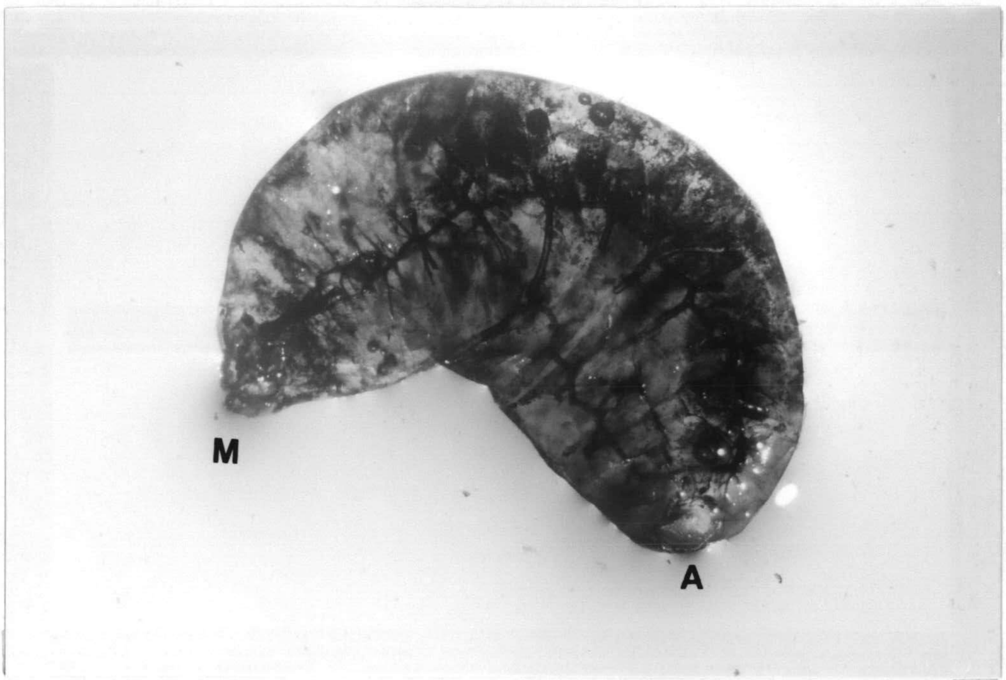


Figure 38. A whole mount of 4th larva was photographed by stereo microscope (SM) magnification at 0.67.

Figure 39. A whole mount of 4[th] larva was photographed by LM magnification objective lens ×4. That showed AP activity at tracheae and integument.

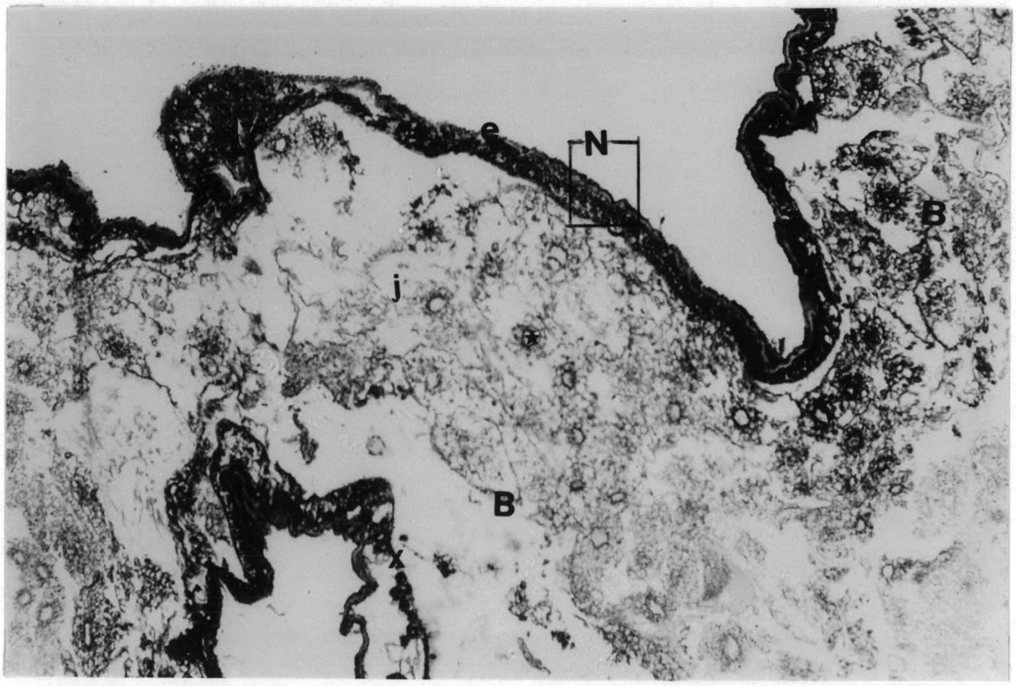


Figure 40. Section of 4[th] larva was photographed by LM magnification objective lens ×10. That showed AP activity at integument and fatty body cell.

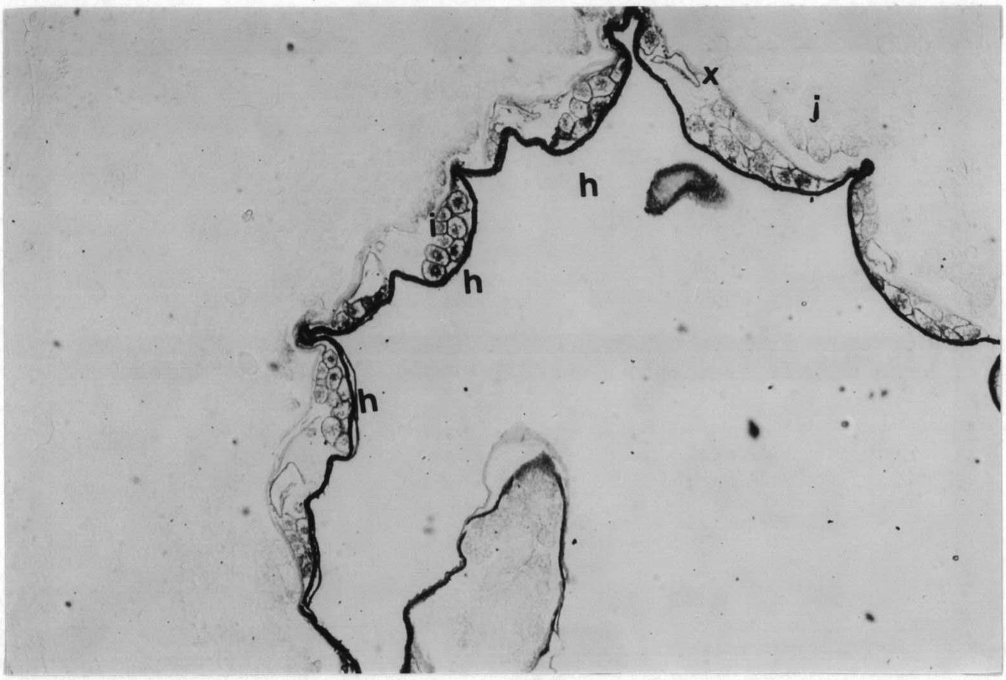


Figure 41. A section of 4^{th} larva was photographed by LM magnification objective lens ×4. That showed AP activity at epithelial cell of limb buds.

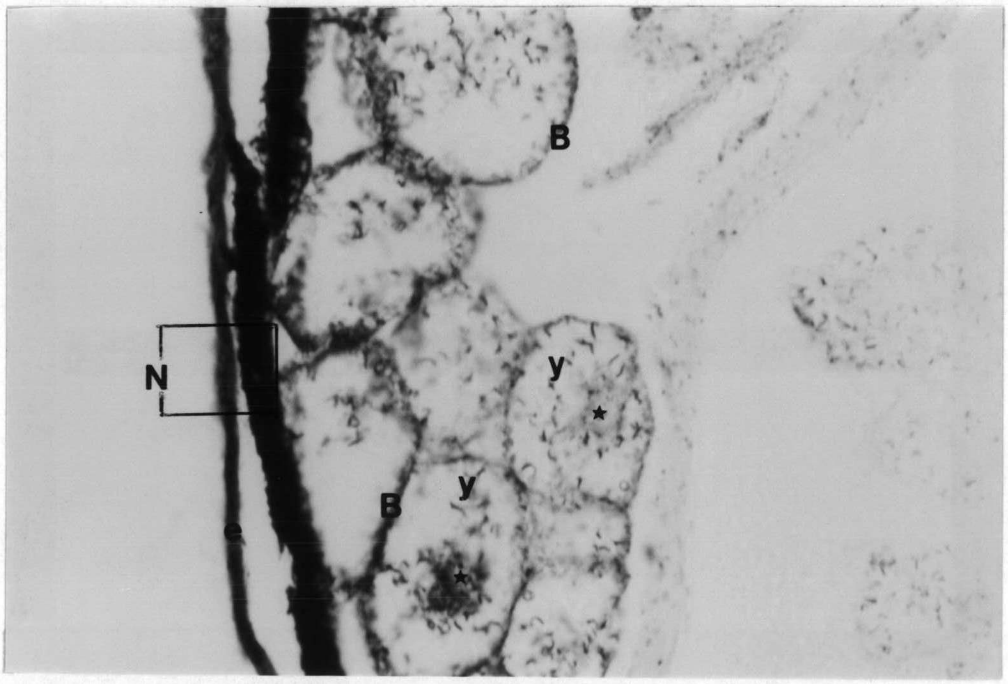


Figure 42. Section of 4^{th} larva was photographed by LM magnification objective lens ×40. That showed AP activity at cuticle and epithelial cell at ventricle.

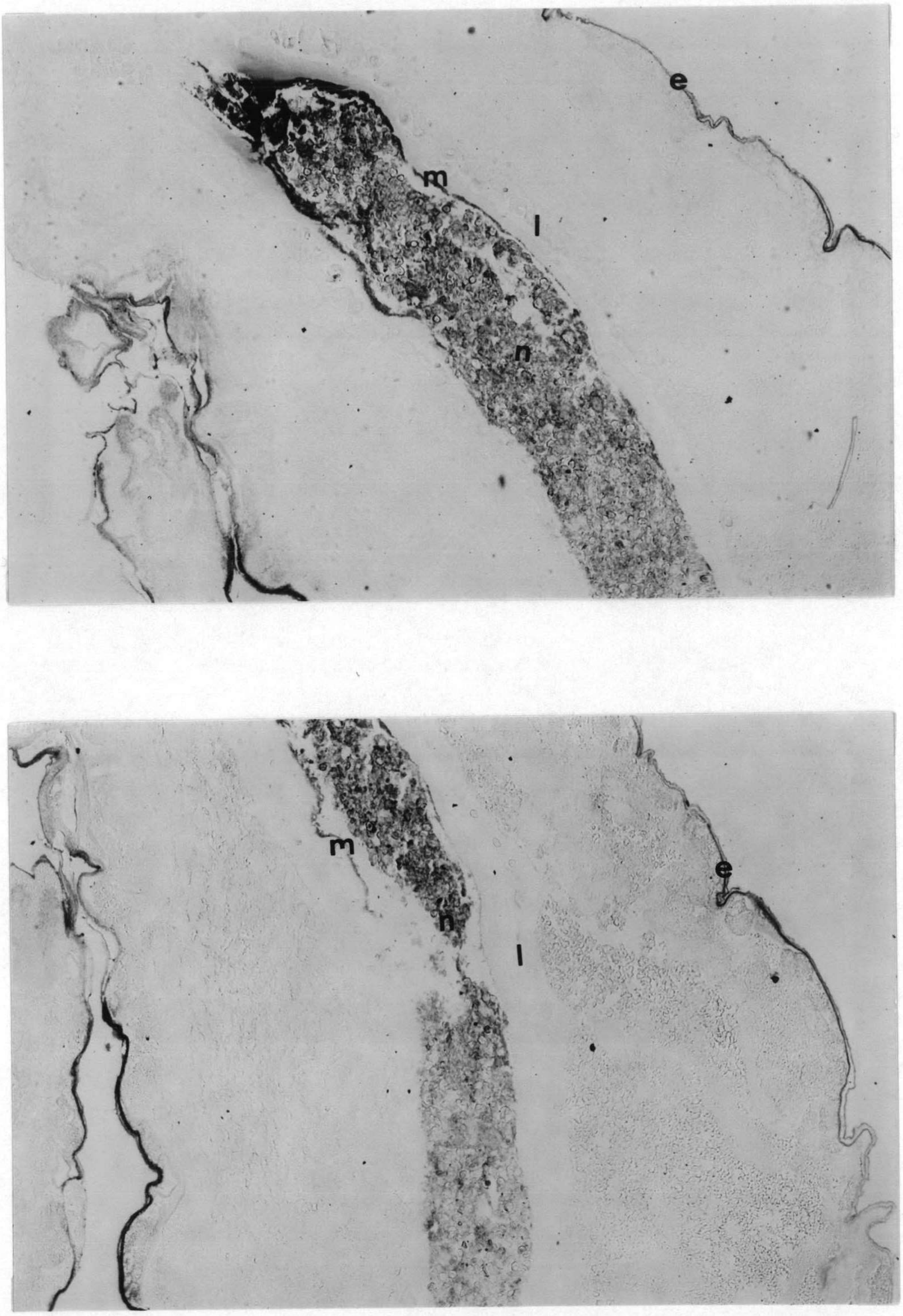


Figure 43. A section of 4th larva was photographed by LM magnification objective lens ×4. That showed AP activity in gut.

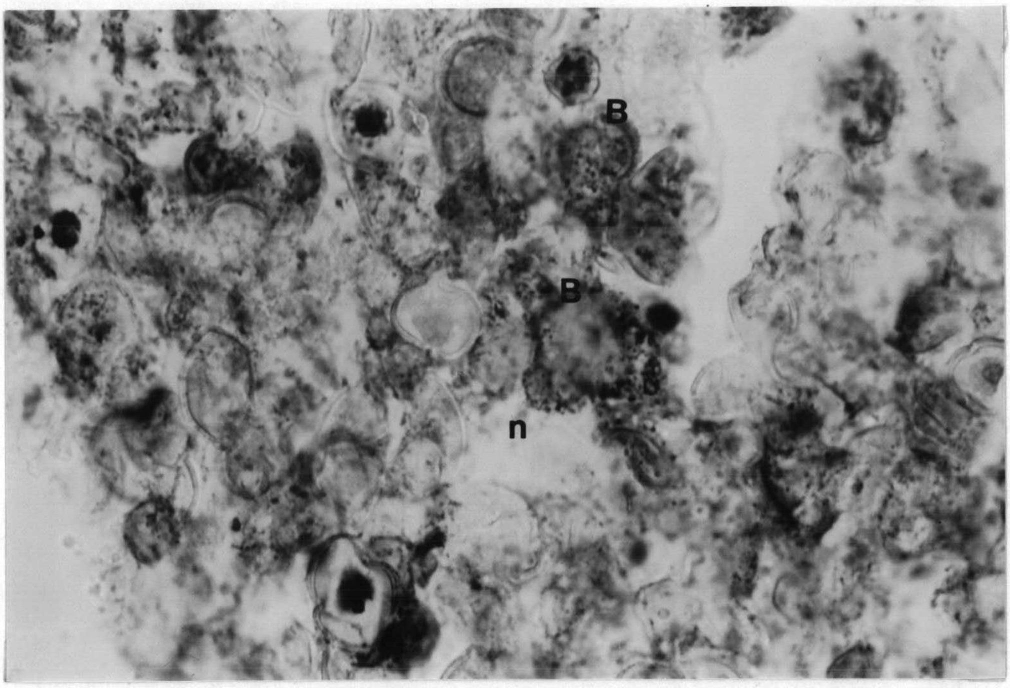


Figure 44. Free AP in gut of 4th larva was photographed by LM magnification objective lens ×40.

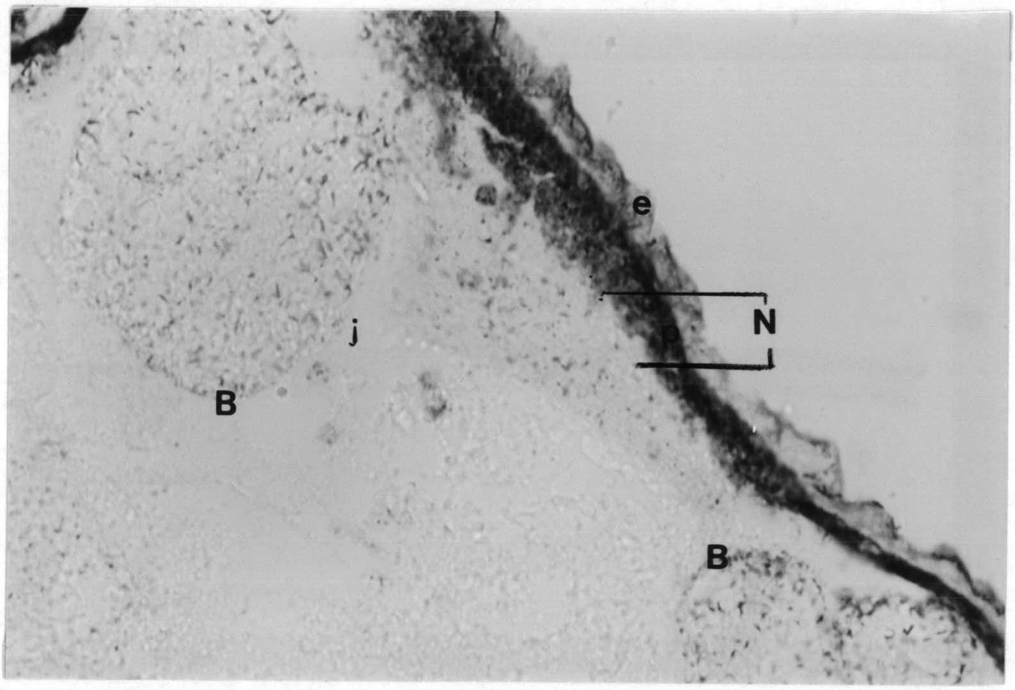


Figure 45. A section of 4th larva was photographed by LM magnification objective lens ×10. That showed AP activity at cuticle and fatty body at dorsal part.

Figure 46. A whole mount of 5th larva was photographed by SM magnification $\times 0.67$.

Figure 47. A section of 5th larva was photographed by LM magnification objective lens $\times 4$. That showed AP activity at cuticle and fatty body.

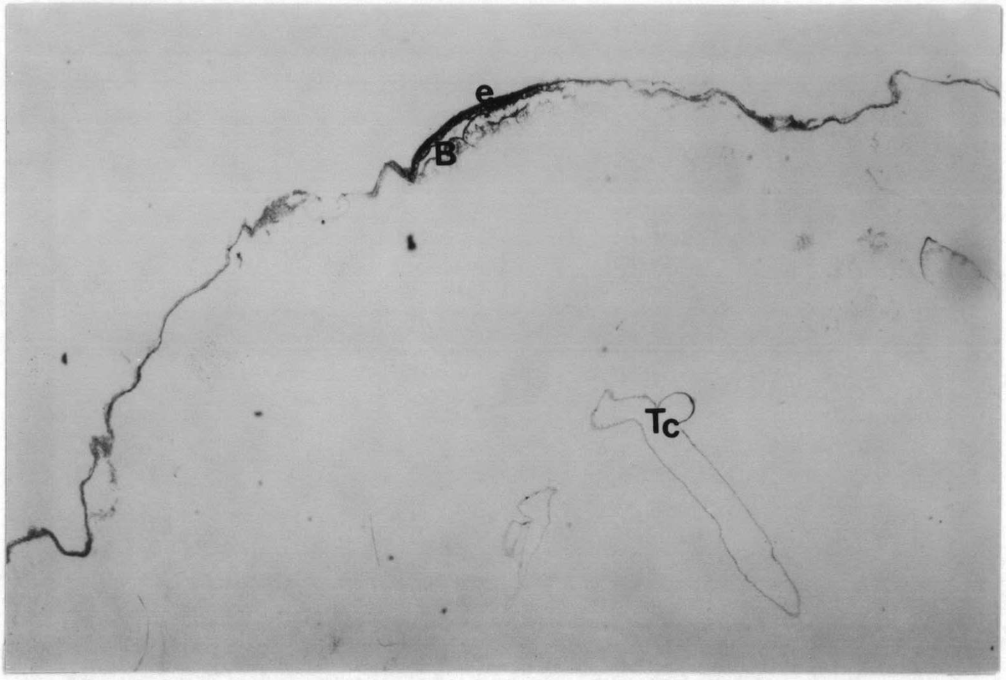


Figure 48. AP activity at tracheae section of 5^{th} larva was photographed by LM magnification objective lens ×4.

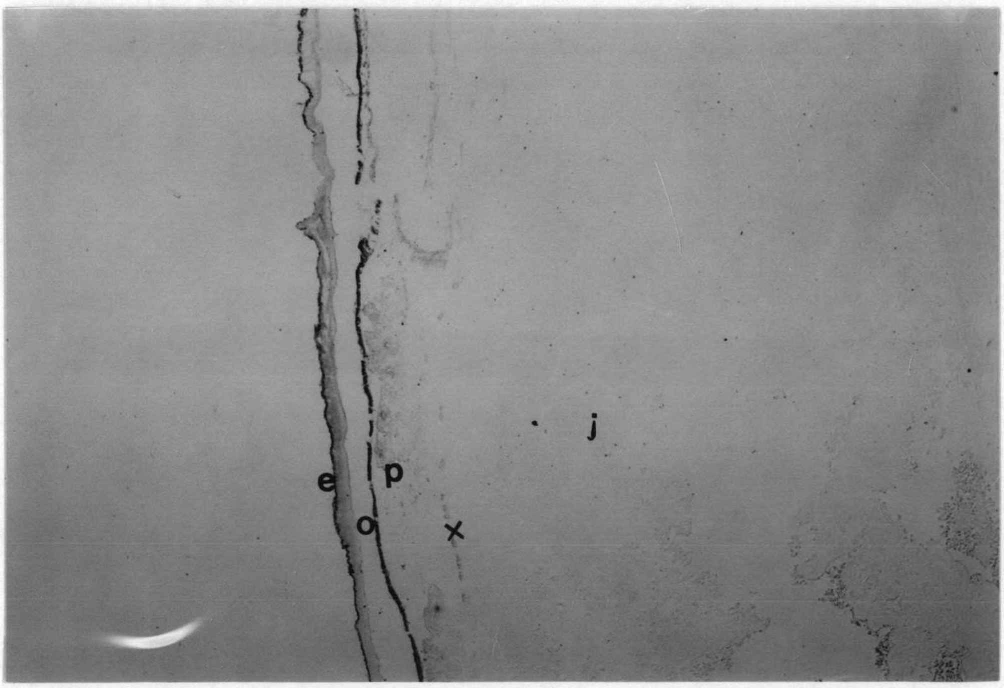


Figure 49. AP activity at integument in 5^{th} larva section was photographed by LM magnification objective lens ×4.

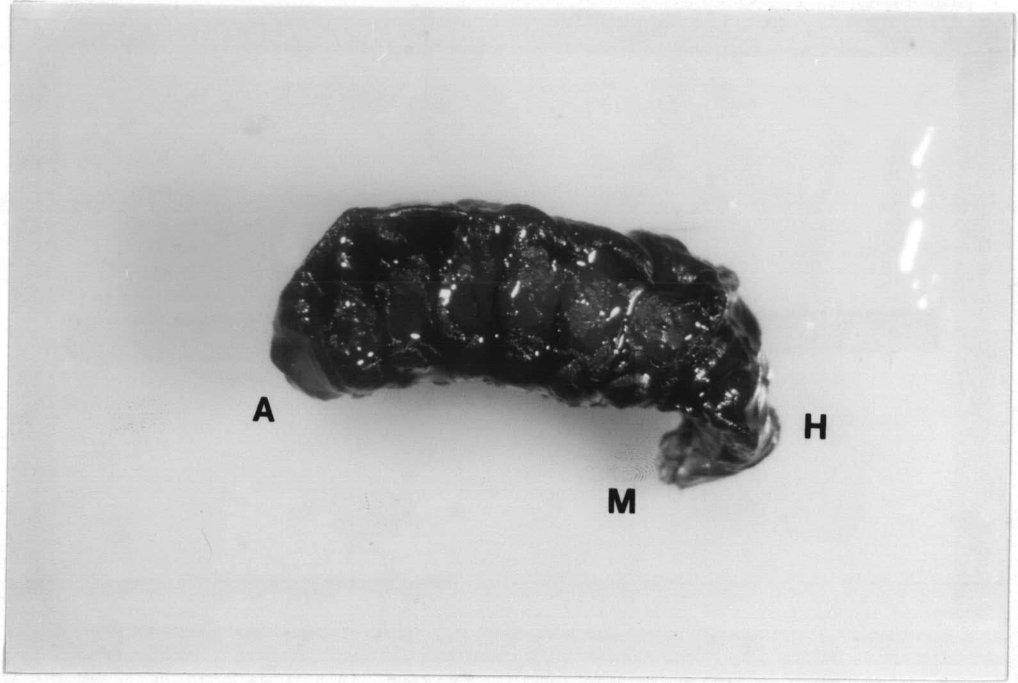


Figure 50. A whole mount of 6th prepupa was photographed by SM magnification objective lens ×0.67.

Figure 51. AP activity at protoduem that develop to head of 6th prepupa was photographed by LM magnification objective lens ×4.

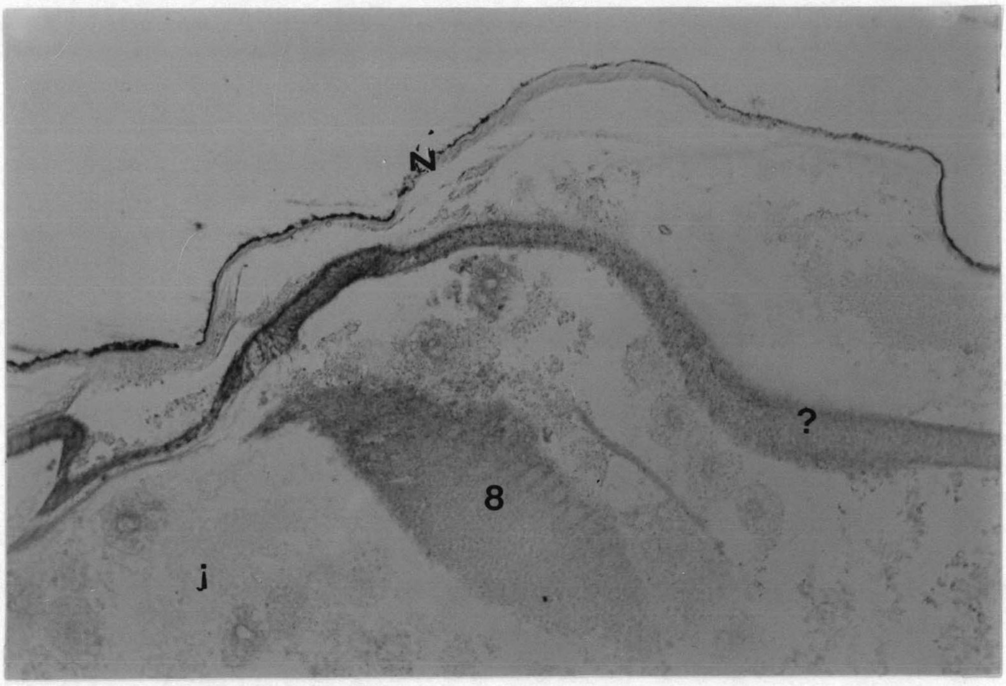


Figure 52. AP activity at integument, 3-4 segment of 6^{th} prepupa section was photographed by LM magnification objective lens ×10.

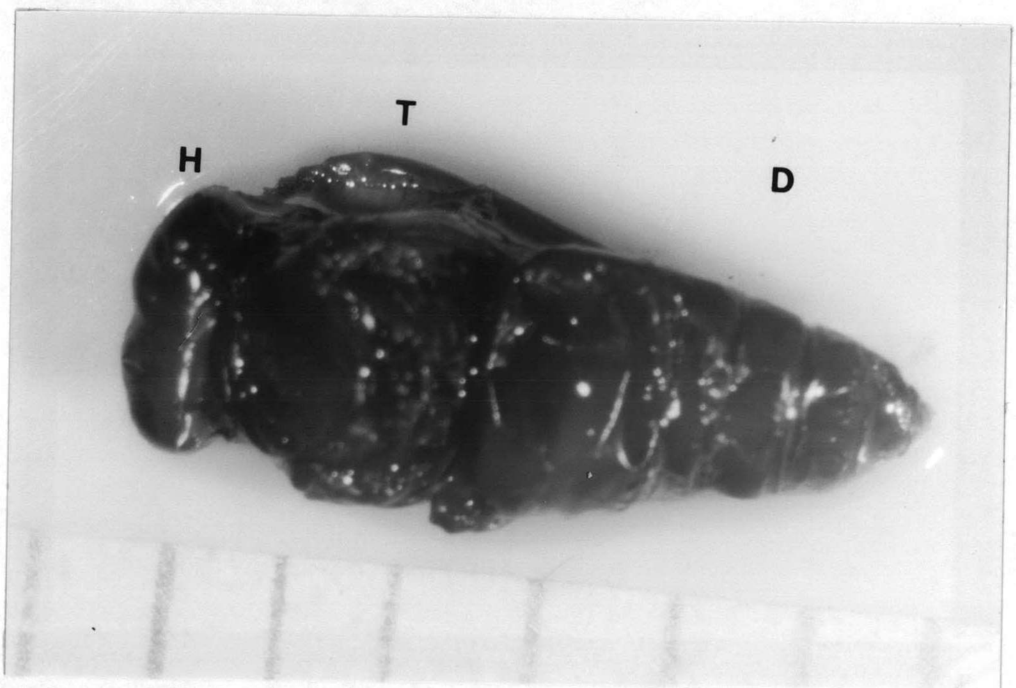


Figure 53. A whole mount of 7^{th} pupa was photographed by SM magnification objective lens ×0.67.

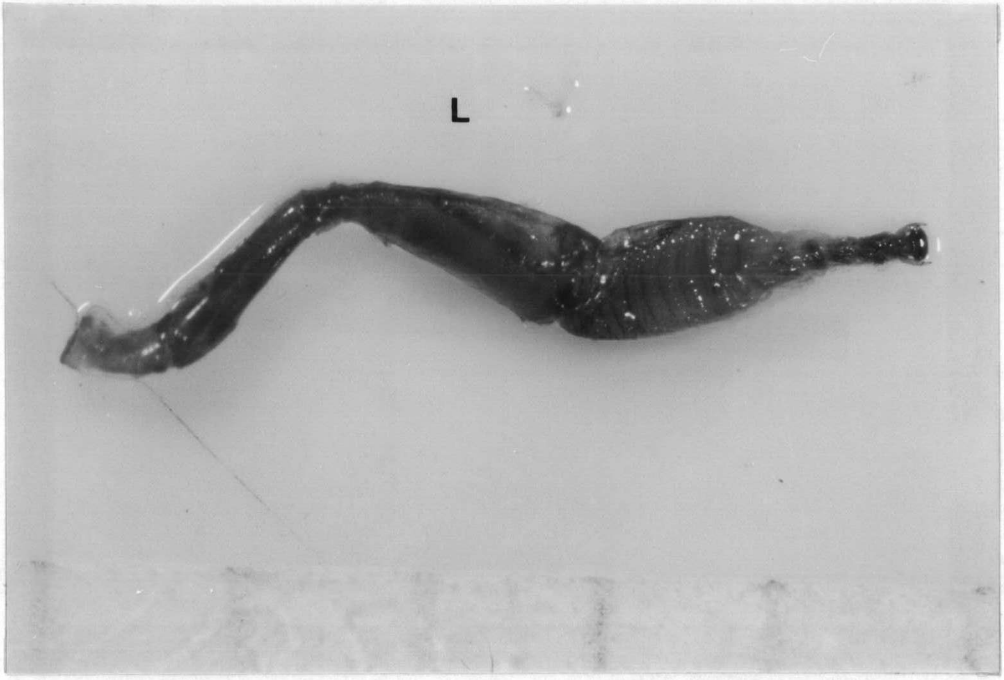


Figure 54. AP activity in legs of 7^{th} pupa was photographed by SM magnification objective lens ×0.67.

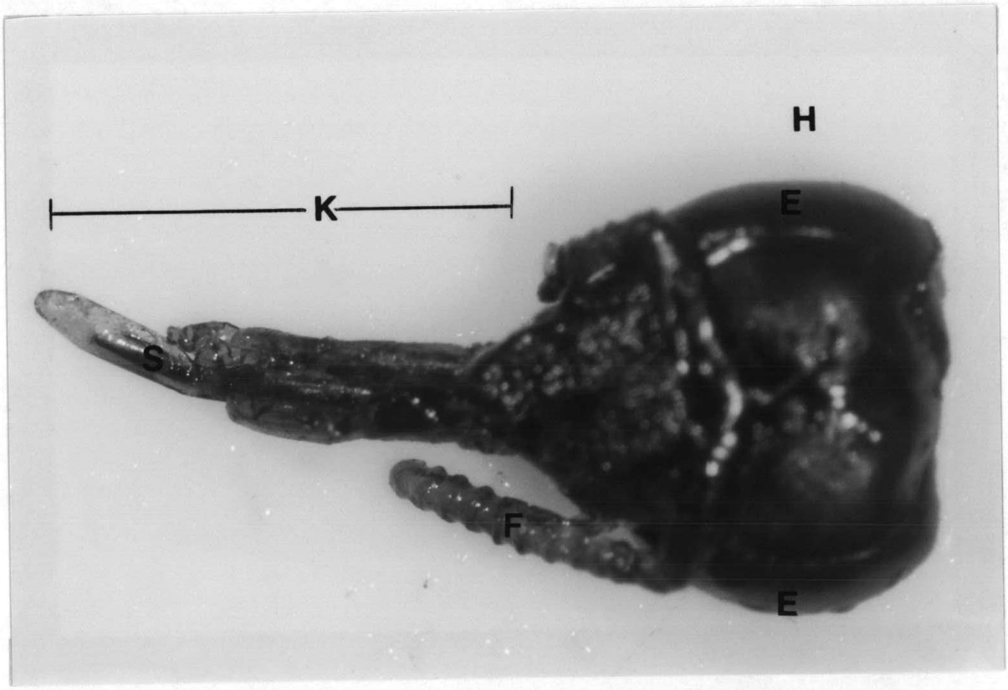


Figure 55. AP activity at head, mouthpart, and antenna of 7^{th} pupa was photographed by SM magnification objective lens ×0.67.

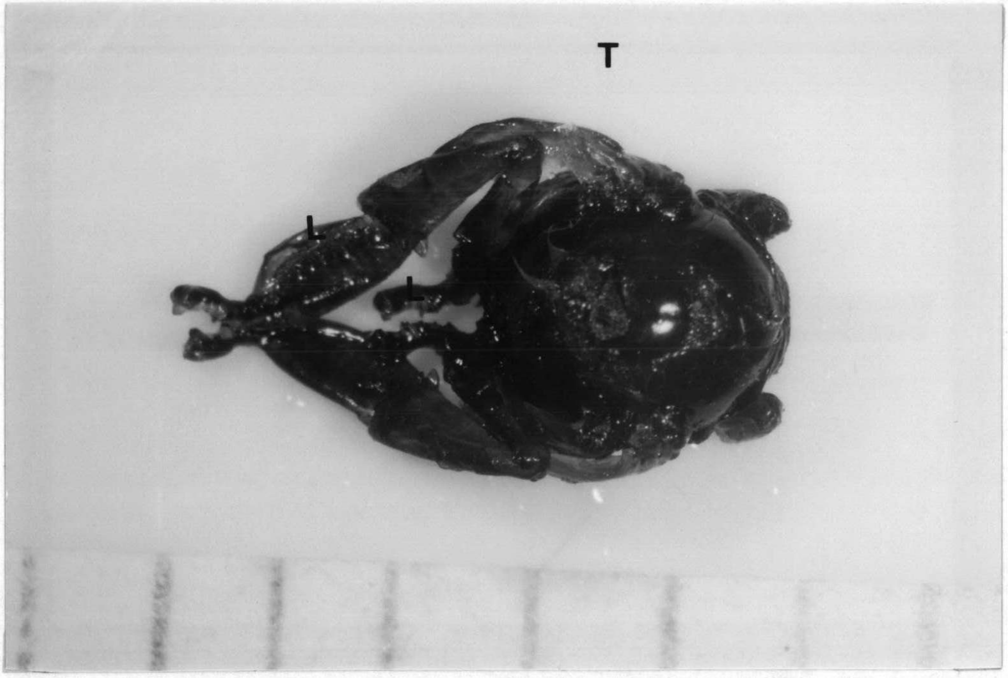


Figure 56. AP activity at thorax of 7th pupa was photographed by SM magnification objective lens ×0.67.

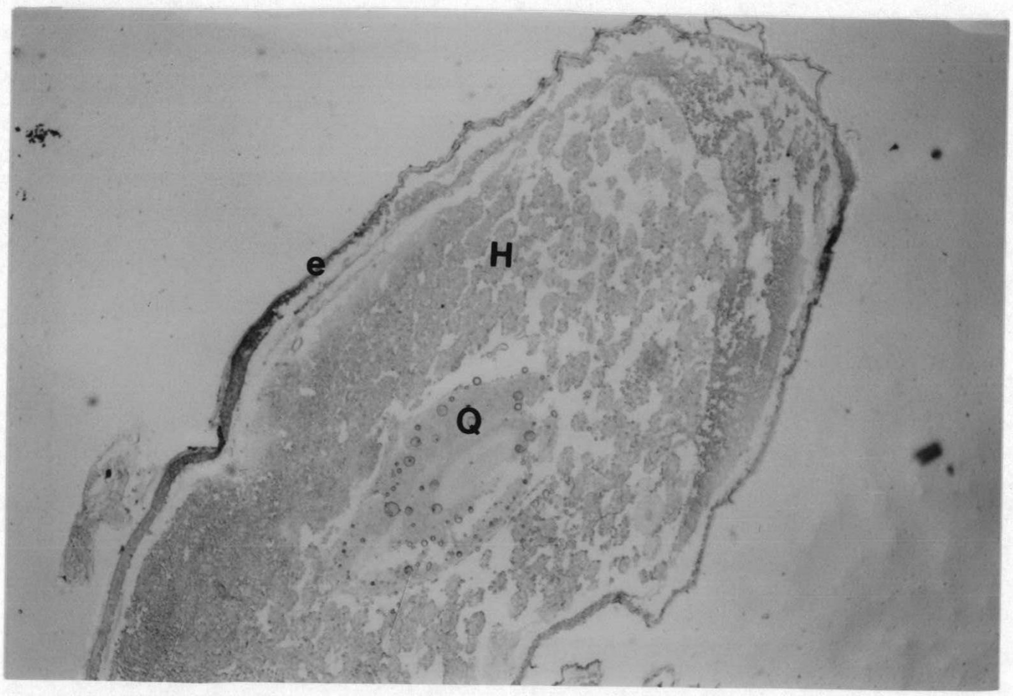


Figure 57. A section of 7th pupa head was photographed by LM magnification objective lens ×4.

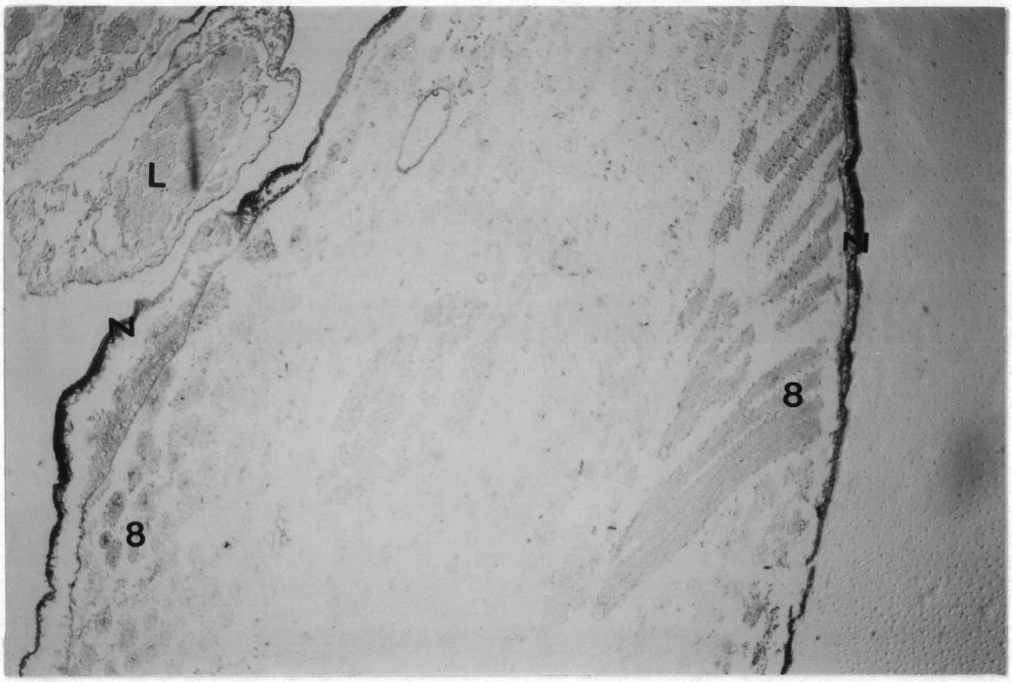


Figure 58. A section of 7^{th} pupa thorax was photographed by LM magnification objective lens ×4. That showed AP activity at muscle cell and integument.

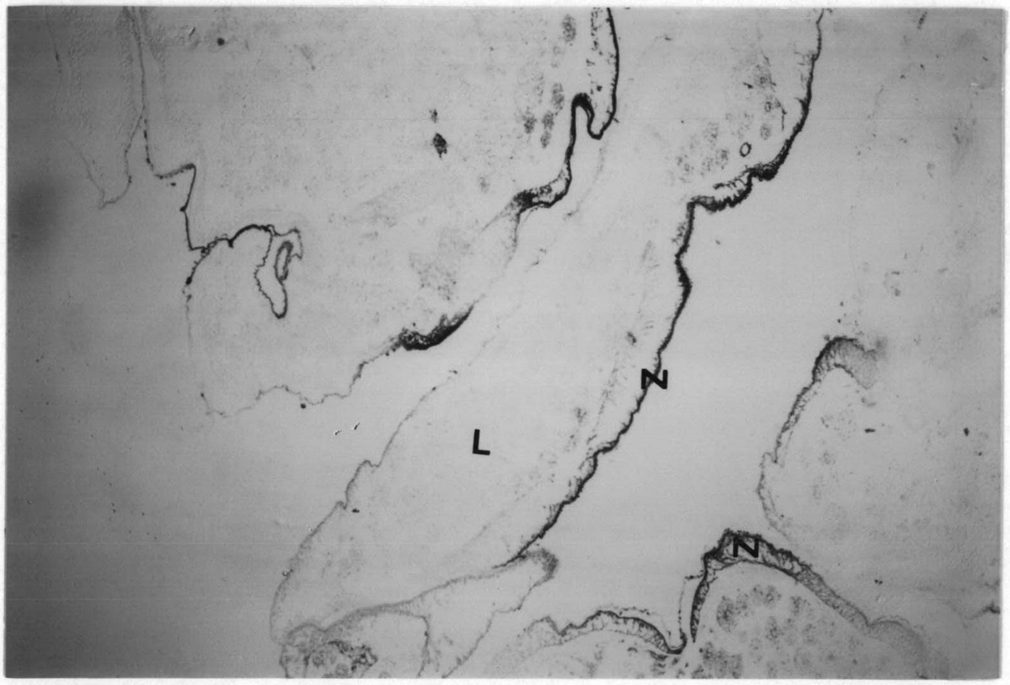


Figure 59. A section of 7^{th} pupa legs was photographed by LM magnification objective lens ×4.

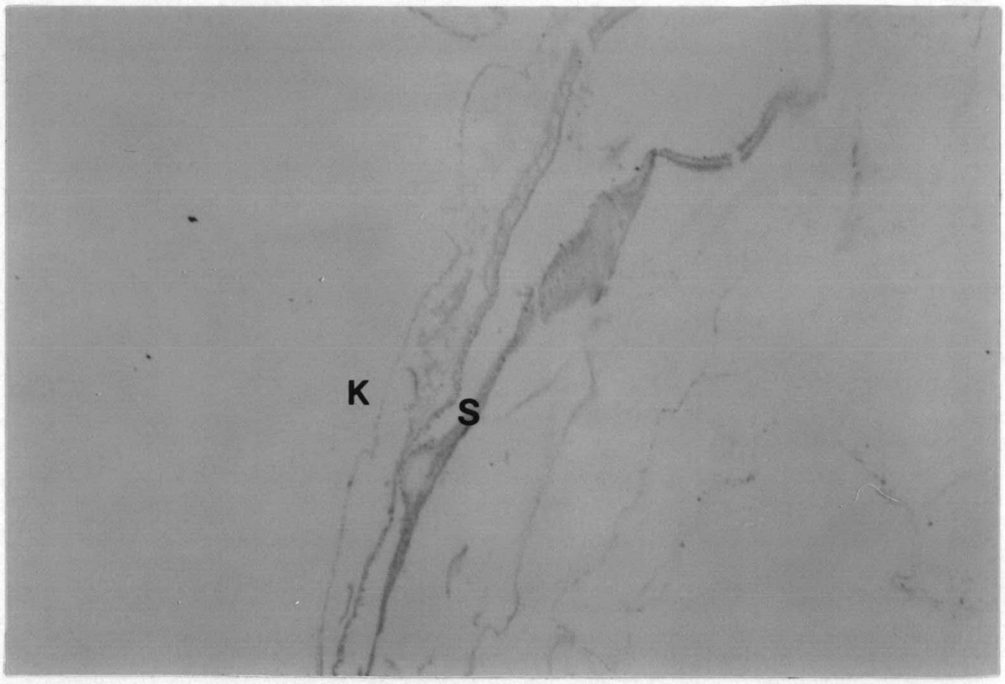


Figure 60. A section of 7^{th} pupa mouthpart was photographed by LM magnification objective lens ×4. That showed AP activity at probosis.

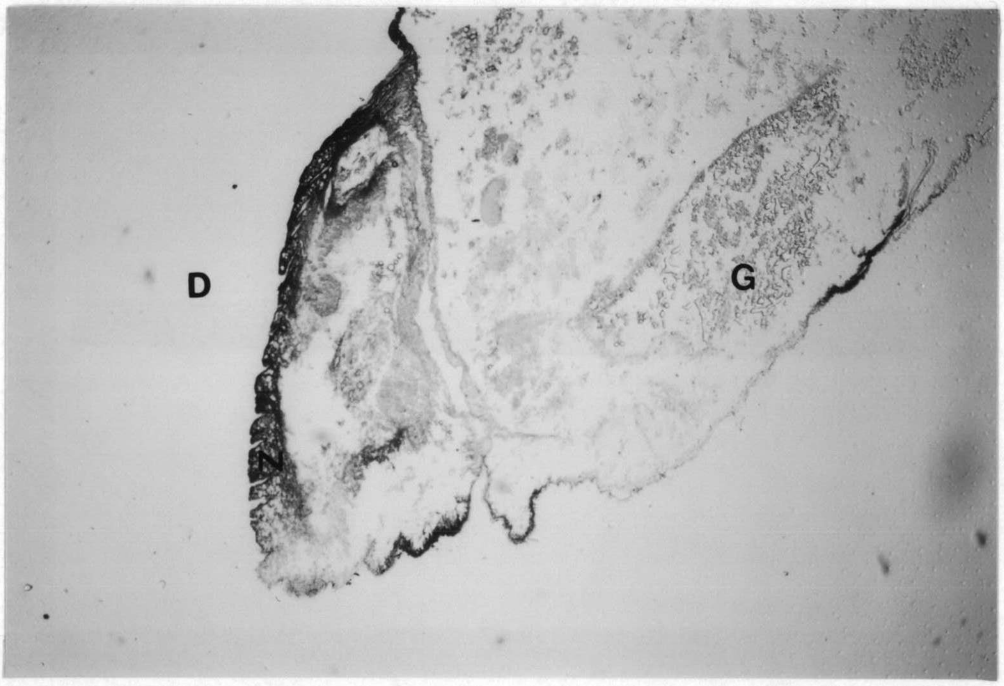


Figure 61. A section of 7^{th}pupa abdomen was photographed by LM magnification objective lens ×4. That showed AP activity at integument and mulpighian tubule

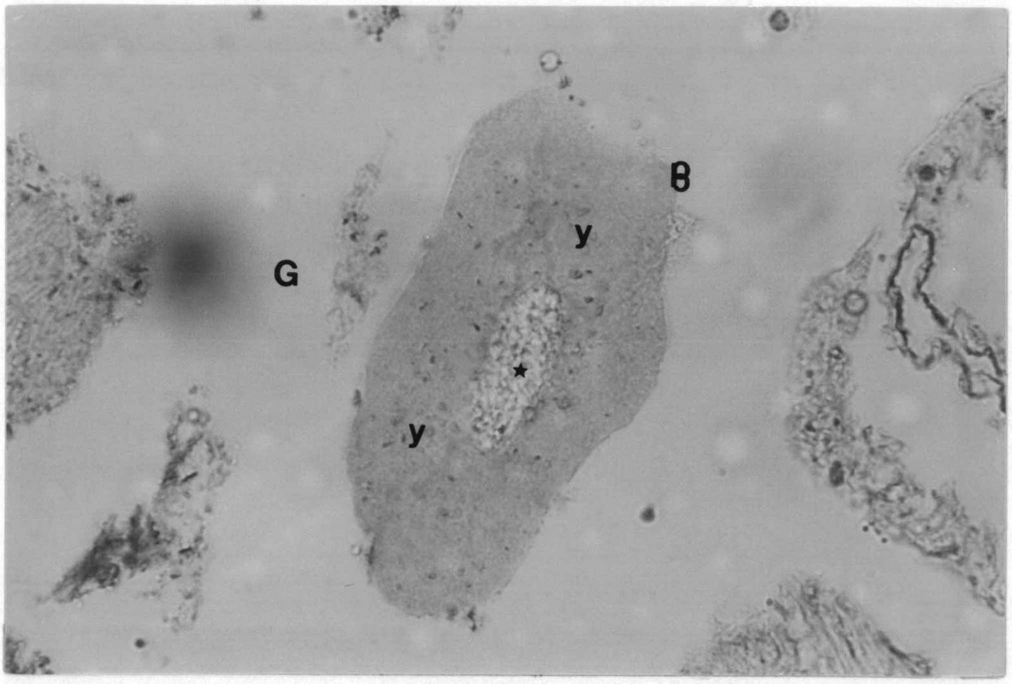


Figure 62. AP activity in free cell in haemocoel of abdomen tissue of 7th pupa was photographed by LM magnification objective lens ×40.

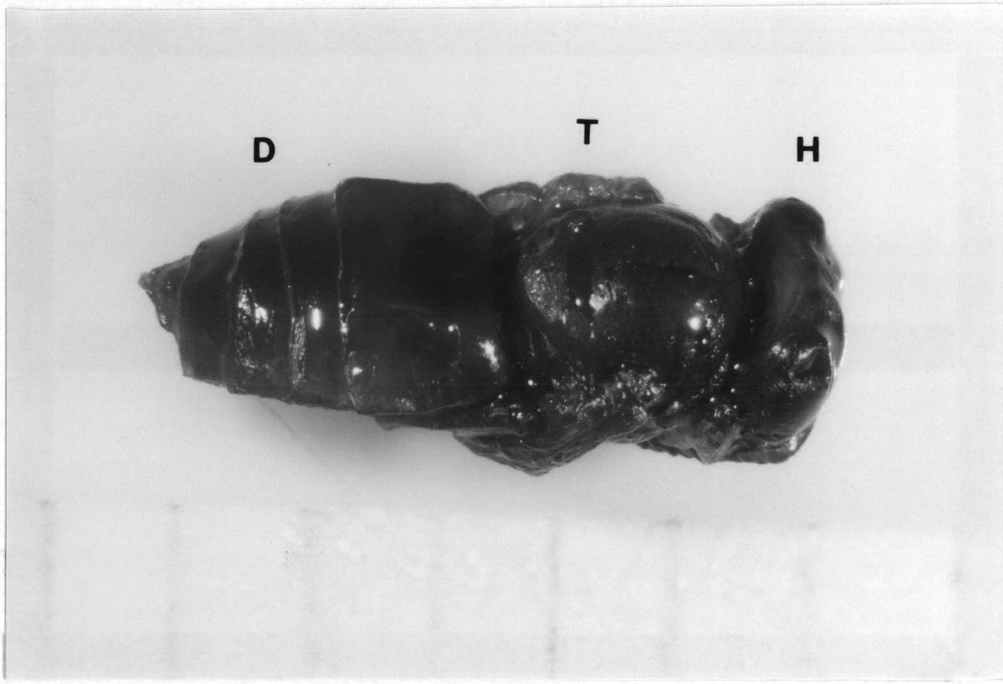


Figure 63. A whole mount of 8th pupa was photographed by SM magnification objective lens ×0.67.

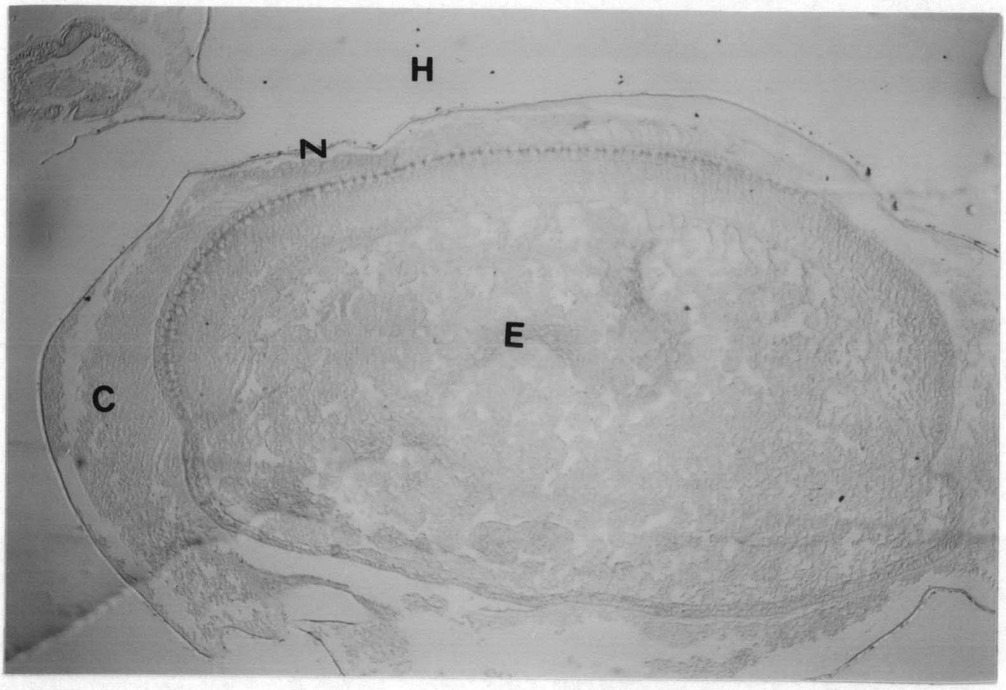


Figure 64. A section of 7^{th} pupa head was photographed by LM magnification objective lens ×4.

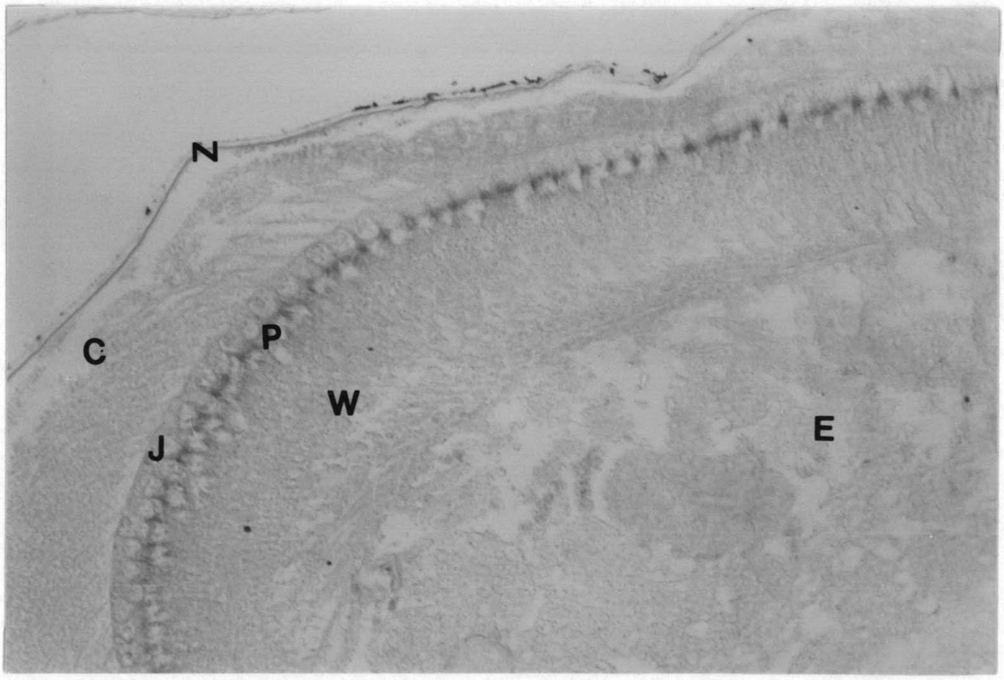


Figure 65. A section of 7^{th}pupa head was photographed by LM magnification objective lens ×10. That showed AP activity at optic lobe.

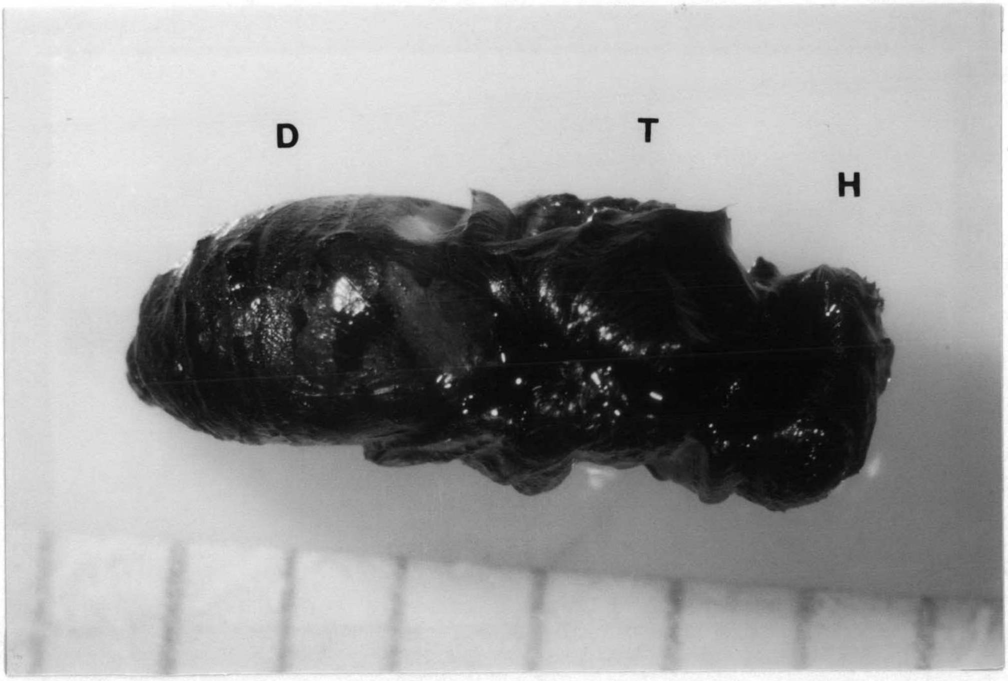


Figure 66. A whole mount of 9th pupa was photographed by SM magnification objective lens ×0.67.

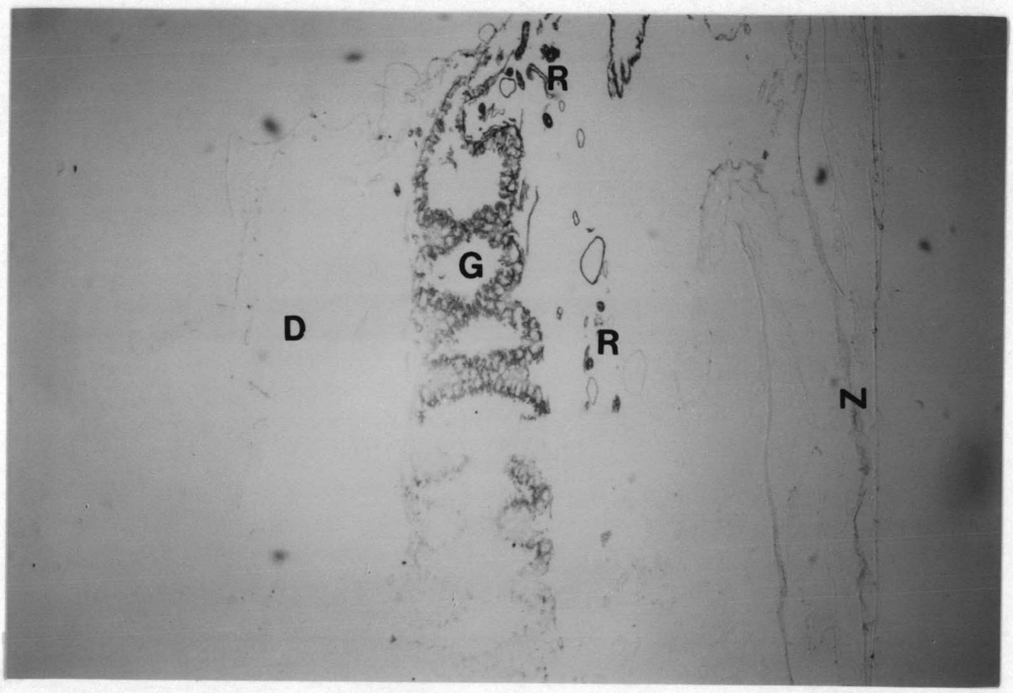


Figure 67. AP activity in epithelial cell of gut L-section of 9th pupa abdomen was photographed by light microscope (LM) magnification objective lens ×4.

Figure 68. AP activity in epithelial cell of 9^{th} pupa was photographed by LM magnification objective lens ×10.

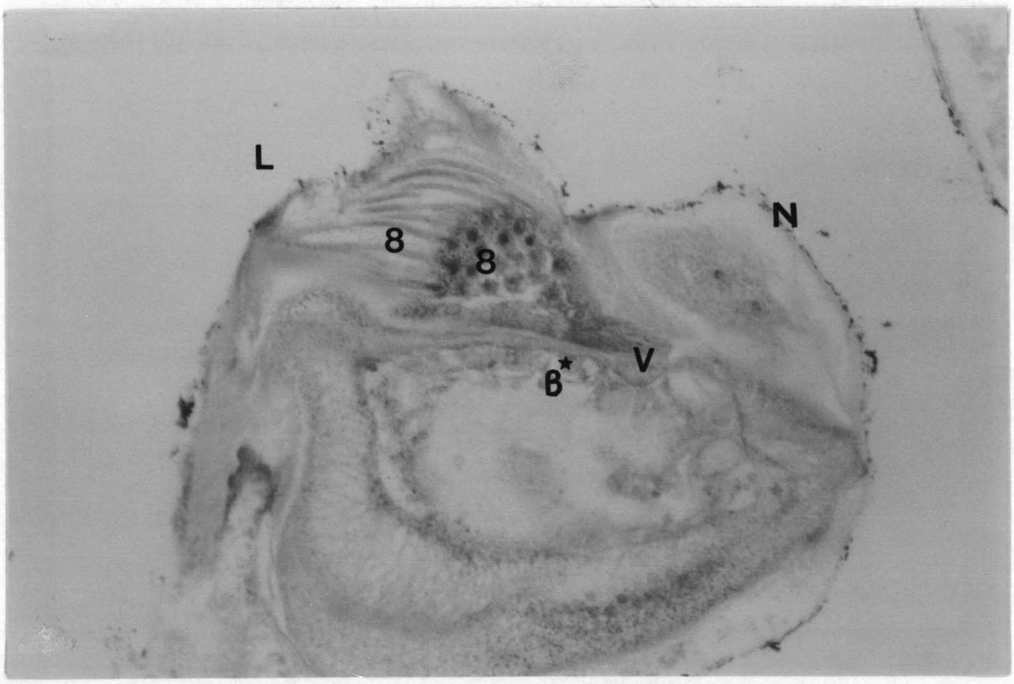


Figure 69. A section of 9^{th} pupa leg joint was photographed by LM magnification objective lens ×10. That showed AP in muscle cell.

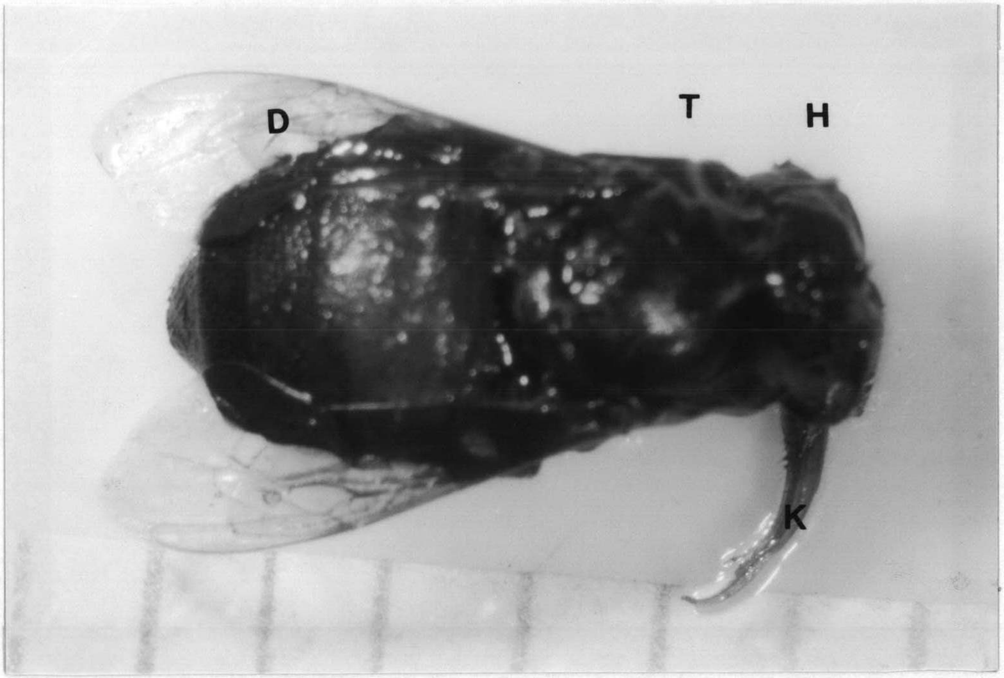


Figure 70. A whole mount of 10[th] emerging adult was photographed by SM magnification objective lens ×0.67.

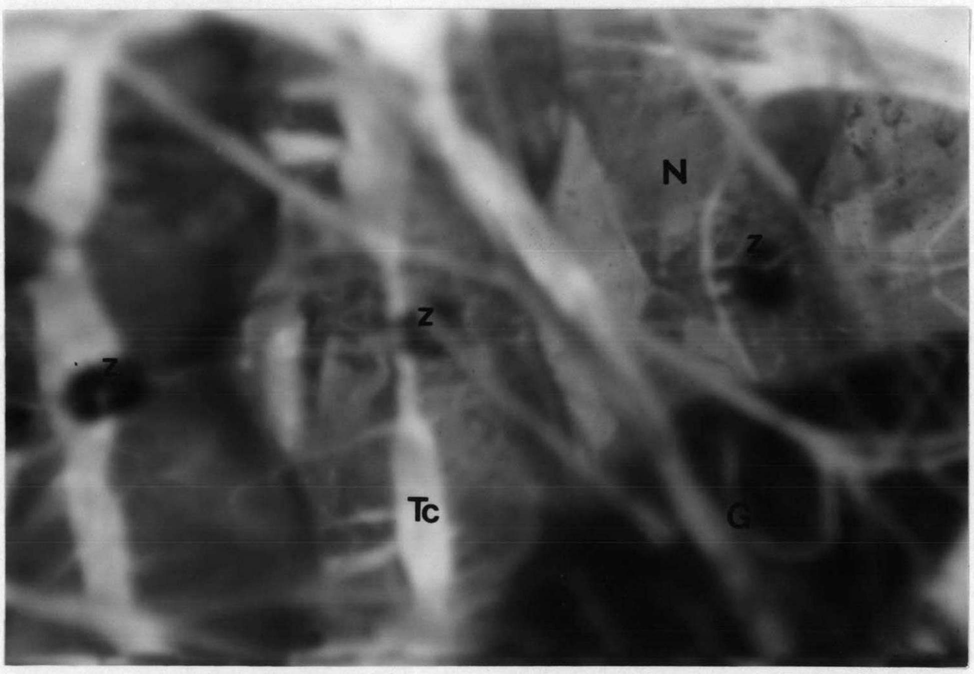


Figure 71. Abdomen of 10[th] emerging adult was photographed by SM magnification objective lens ×0.20. That showed AP activity in ganglia at abdomen.

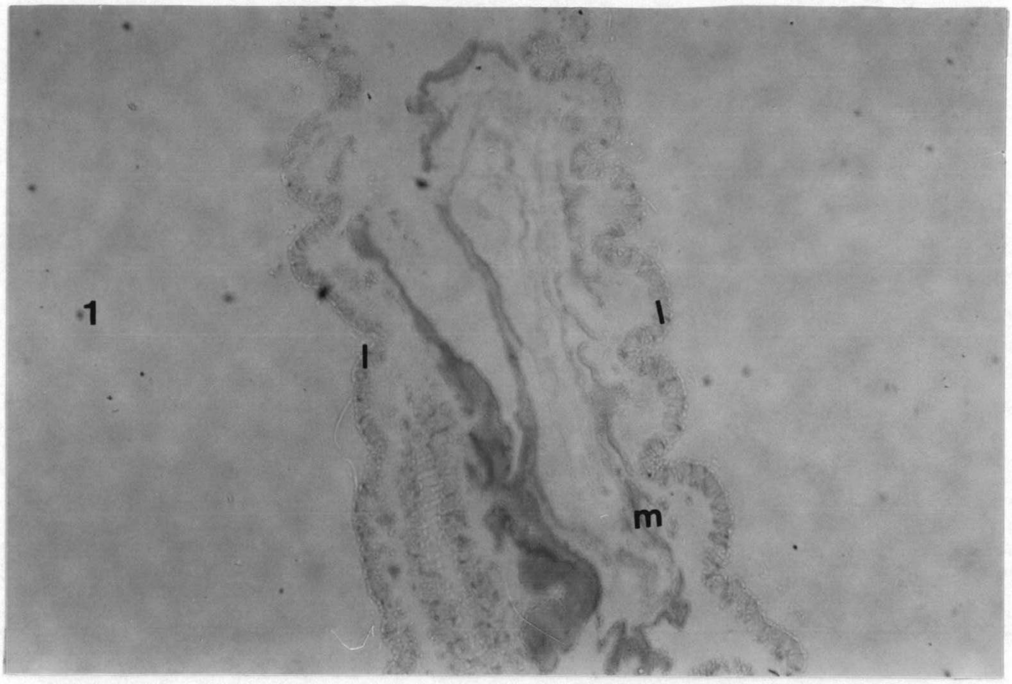


Figure 72. AP activity in foregut of a L-section at 10^{th} emerging adult was photographed by LM magnification objective lens ×40.

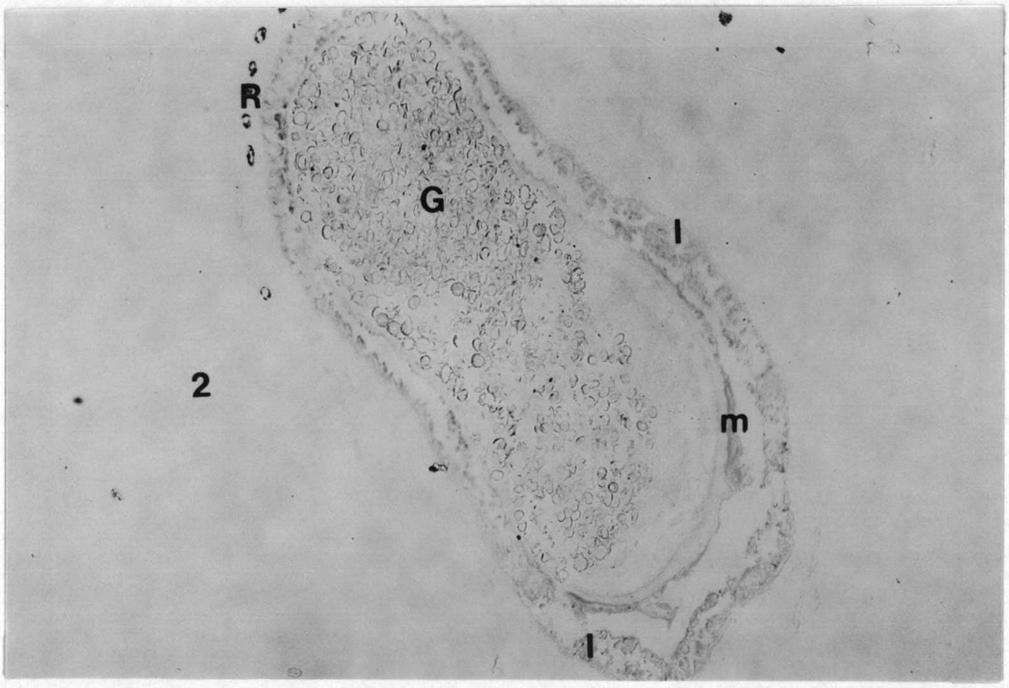


Figure 73. AP activity in midgut of X-section at 10^{th} emerging adult was photographed by LM magnification objective lens ×10.

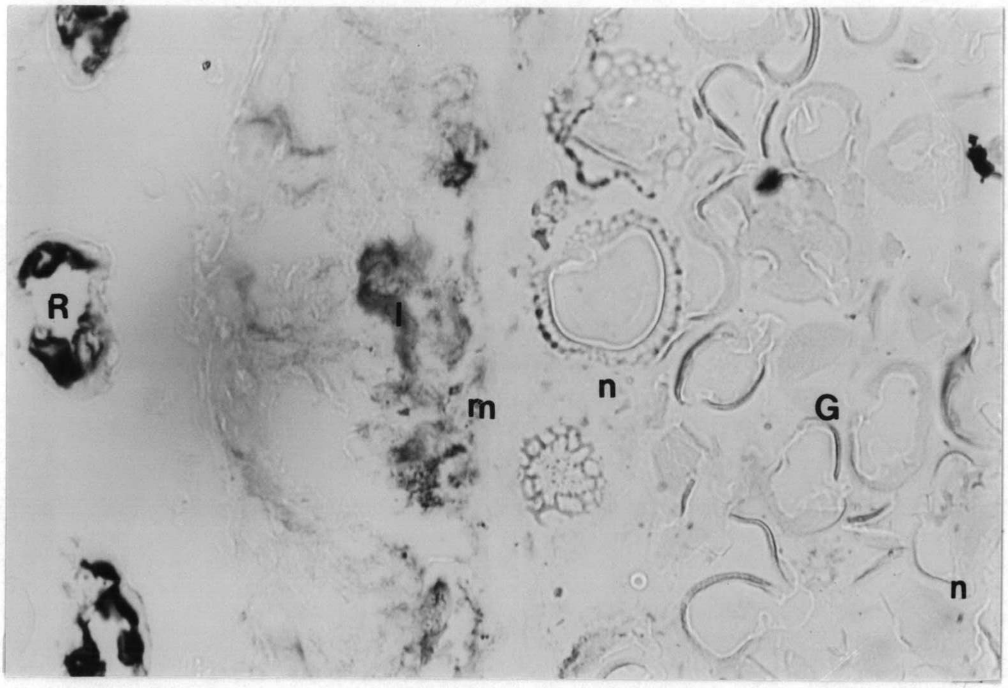


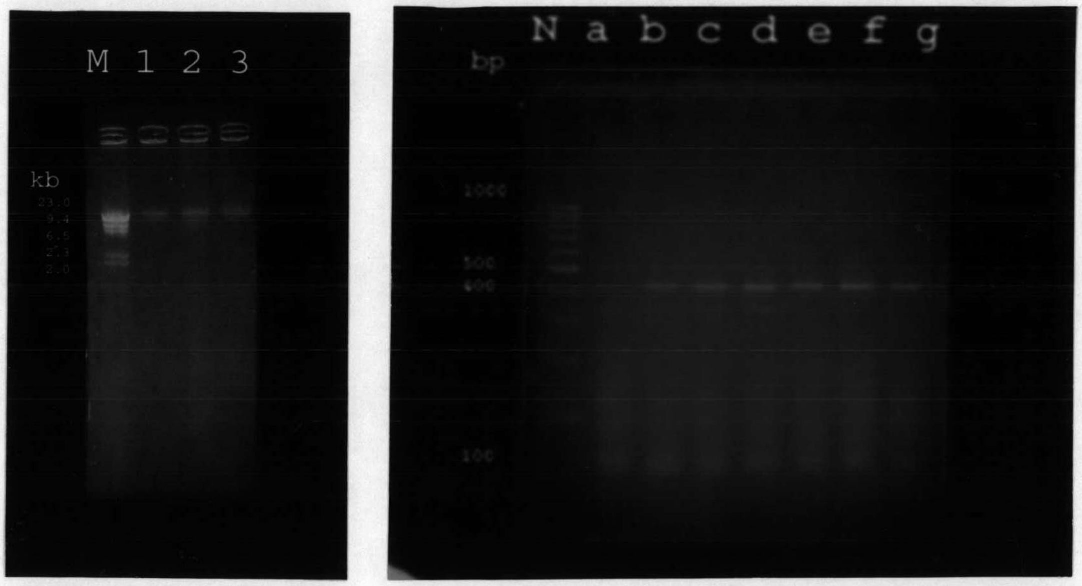
Figure 74. AP activity in midgut of X-section at 10^{th} emerging adult was photographed by LM magnification objective lens ×40.

3. Partial DNA sequence of *AP* in *A. mellifera*

Genomic DNA extraction, PCR amplification, PCR purification, and sequence analysis

The size of genomic DNA of *A. mellifera* was approximately 23 Kb (Figure 75A). Then, genomic DNA was amplifield by AP1 and AP2 primers under the optimum condition. Both of AP1 and AP2 primers were converted from VPDSAGTAT and VEGGRID amino acid sequences of *Aph-4* in *D. melanogaster*. These partial amino acid sequences were highly conserved regions. The nucleotide sequence of AP1 primer (forward primer) was 5' GTG CCG GAT TCG GCG GGC ACT GCC ACT 3' (27 nucleotides long). The nucleotides sequence of AP2 primer (reverse primer) was 5' GTC AAT GCG ACC TCC TTC CAC 3' (21 nucleotides long). A PCR reaction was prepared by 1 μl of genomic DNA, 0.4 μM AP1 and AP2 primers each, 25 mM dNTPs, 1.5 mM $MgCl_2$, 1X PCR buffer, and 1 unit of Taq DNA polymerase. The PCR condition was at 94°C for 2.5 min and 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 3 min. The last extension was at 72°C for 10 min. The length of PCR product was about ~ 420 bp (Figure 75B). After that, PCR was purified to clone with TOPA TA cloning. Then, clone was selected and purified before sequencing. From sequencing data, the length of obtained partial nucleotides was 429 bp (Figure 76A). It was aligned with part of nucleotide sequences of *D. melanogaster Aph-4*, membrane-bound *AP* of *B. mori*, non specific *AP* in *G. gallus*, Bovine, and *H. sapiens* at www.ebi.ac.uk/emboss/align.html. The result shows the nucleotide similarity to *D. melanogaster* at 39.2%, to *B. mori* at 39.7% , to non specific AP in *G. gallus* at 36.6%, to non specific AP in Bovine at 36.9%, and to non specific AP in *H. sapiens* at 36.7%. The obtained nucleotide sequence was converted to an amino acid sequence of 139 amino acids at www.ebi.ac.uk/contrib/tommaso/translate.html (Figure 76B). From alignment analysis of amino acid sequence, high conserved primer regions (VPDSAGTAT and VEGGRID) were observed. The similarity of AP amino acid sequence was at 14.3% of *D. melanogaster*, at 24.3% to *B. mori*, at 28.6 % to *G. gallus*, at 19.8% to Bovine, and at 27.6% to *H. sapiens*.

Both nucleotide and amino acid sequences were compared by the multiple sequence analysis program. That was Mutalin of NIRA database at www.toulouse.inra.fr/centre/centre/multalin.html, The result showed conserved sequence to *AP* in several organisms (figure 77 and 78). Otherwise, multiple sequence analysis program was (ClastalW) of EMBL database at www.ebi.ac.uk/services/clastalw. Nucleotide and amino acid sequences was analyzed. Clustal W showed concensus region and phylogenetic tree (Figure 79).



A B

Figure 75. The quality of genomic DNA was determined by the sharp and high molecular weight band on 1% agarose gel (A). PCR product of 429 bp was obtained from genomic DNA amplification by AP1 and AP2 primers (B).

M	represents	λ*HindIII* marker
1, 2, and 3	represents	genomic DNA
N	represents	100 bp marker
a, b, c, d, e, f, g	represents	PCR product at differential condition of PCR amplification

A

AP1

```
5'   GTG CCG GAT TCG GCG GGC ACT GCC ACT CTC   30
     GGA TGA AAA GGC GCG CAC ATA TCG GGA AAG
     GCG CGA TCG TGG CTC AAA GAT TAC GAA TTA   90
     GAG GCG GTG AAA ATA ATG GTC GAC GAT CCA
     ACG ATT TAT CGA CGA TCG AAT AAG ATT TAT   150
     CGC GAA TTC TTG CCG CGT TCC AGT TTT TCC
     TTT AAT ATT CCG TGT GCT CGC GGT GTT GGA   210
     TAA GGG ATT GAG GGT GGG ATG GAG GGG AGA
     GGG GTC GCG AGT AAA CTG ACA ACC GAC AGG   270
     ATA TAT AAA TAA ATT CGA CAT CGC GTT GAA
     TCC TTT GGT CAC GAA TGA CGT ATG CAC CGG   330
     AAT GTT TCG AAA TCT TCT TTC TTT TTT TCT
     TTT TTT CTT TTT TCC GAG CGA AAA GTT TCA   390
     ATT CGC TGC AAC GTC GAG TGG AAG GAG GTC
     GCA TTG AC 3'
```

AP2

B

```
VPDSAGTATLG*KGAHISGKARSWLERLRIRGGEN 35
NGRRSNDLSTIE*DLSRILAAFQFFL*YSVCSRCW
IRD*GWDGGERGRE*TDNRQDI*INSTSR*ILWSR 105
MTYAPECFEIFFLFFFFSFFRAKSFNSLQRRVEGG
RID
```

Figure 76. Nucleotide sequence of PCR product in *A. mellifera* alkaline phosphatase that was amplified by AP1 and AP2 primers (blue color). The length of obtained partial nucleotides was 429 bp (A). Amino acid sequences were converted from nucleotide sequence at www.ebi.ac.uk/contrib/tommaso/translate.html (B).

```
            1                                                  50                                                 100                                                150
       Apis GTGCCGGATT CGGCGGGCAC TGCCACTCTC GGATGAAAAG GCGCG....C ACATATCGGG AAA...GGCG CGATCGTGGC T........C AAAGATTACG AATTAGAGGC GGTGAAAATA ATG..GTCGA CGATCCAACG ATTTATCGAC
 Drosophila GTGCCGGATT CGGCGGGCAC TGCCACTGCC ATCTTCTCGG GTTCGAAAAC CCATTACGGA GCCATTGGAA TGGACGCCAC .......... ..CCGCTCCA AG....AAGA ATGGGCAGCA AGGCAGGGTC CAGAGCGTCA TGGAGTGGGC
     Bombyx GTCCCGGACT CGTCGTGCAC GGCGACGGCA TACCTGTGCG GCGTGAAGGC CAACCAAGGC ACCCCTGGAG TGACGGCCGC CGTGCCGAGG CACGACTGCG AGGCTTCCAC CGACGTCACC AAGC.GGGTT CAGTCCATCG CCGAGTGGGC
     Gallus GTGCCCGACA GCGCGGGCAC AGCCACTGCT TACCTCTGCG GCGTCAAAGC CAACGAGGGG ACAGTGGGAG TCAGCGCCGG TGTCACCCGT GACCGCTGCA A..CACCACC AAGGGCCAGG AGGT.GACCT CCATCCTCCG C....TGGGC
     Bovine GTCCCTGATA GCGCAGGCAC CGCCACCGCC TACTTGTGTG GGGTGAAGGC CAATGAGGGC ACCGTGGGGG TGAGCGCAGC AACCCAGCGC TCCCAGTGTA A..CACCACT CAGGGCAACG AGGT.GACCT CCATCCTGCG C....TGGGC
       Homo GTCCCTGACA GCGCCGGCAC CGCCACCGCC TACCTGTGTG GGGTGAAGGC CAATGAGGGC ACCGTGGGGG TAAGCGCAGC CACTGAGCGT TCCCGGTGCA A..CACCACC CAGGGGAACG AGGT.CACCT CCATCCTGCG C....TGGGC
  Consensus GTgCCgGAtt cggCggGCAC tGCcACtgcc ..ctt.t..G GcgcGaa..C ccAt.acGG. acc...GG.g tga.cGc.gC .......... .acga.T.Cg A...a.a.gc .g.gg.aaca A.G..G.c.. C.atcCa.Cg .....TgGgC

            151                                                200                                                250                                                300
       Apis GATCGAATAA G..ATTTATC GCGAATTCTT GCCGCGTTCC AGTTTTTCCT TTAATATTCC GTGTGC.... .TCGCGGTGT TGGATAA..G GGATTGAGGG TGGGATGGAG GGGAGA.... .......... .GGGGTCGCG AGTAAACTGA
 Drosophila CCAGAAGGAG GGCAAGCGCA CCGGCGTGGT CACCACAACC AGGATCACGC ACGCCACGCC CGCCGCCACC TACGCCACAT CTAC.GACCG GGACTGGGAG TGCGACACGG AAGTGCCCGC GGA....ATC GGTGGGCTTT CATGTC..GA
     Bombyx ACTGGCTGAC GGGAGAGATG TCGGTATAGT GACGACCACT CGCATCACGC ATGCCTCTCC GGCGGGCACT TTCGCCAAGG TCGCCAACAG GAACTGGGAG AATGATAACG ACGTGAAACA AGAAGGCCAC GACGTCAACC GCTGTCCGGA
     Gallus TAAGGATGAA GGCAAGGCGG TGGGCATTGT CACCACCACG CGGGTGACCC ACGCGACACC CAGCGCTGCC TACGCGCACT CAGCCAACCG TGACTGGTAC TCAGATGGAG AGATGCCCCT GGA.TGCACT GG..AGGGCG GCTGCAAAGA
     Bovine CAAGGACGCA GGGAAATCCG TGGGCATCGT GACCACCACG CGCGTGAACC ACGCCACCCC CAGCGCCTCC TACGCCCACT CTGCTGACCG GGACTGGTAC TCGGACAACG AGATGCCCCC GGA.GGCCCT GA..GCCAGG GCTGCAAGGA
       Homo CAAGGACGCT GGGAAATCTG TGGGCATTGT GACCACCACG AGAGTGAACC ATGCCACCCC CAGCGCCGCC TACGCCCACT CGGCTGACCG GGACTGGTAC TCAGACAACG AGATGCCCCC TGA.GGCCTT GA..GCCAGG GCTGTAAGGA
  Consensus .atgga.gaa Gg.A..tat. .cGg..T.gT gaCgac.aCc aG..T.accc atgccactCC g.g.Gcc.c. ttCGCc..gt t.gctaAc.G GgAcTGggag tg.GAta..G aggtGa.... .ga....... gg.gg.c.cg ..Tg.ac.GA

            301                                                350                                                400                                                450
       Apis CAACCGACAG GATATATAAA TAAATTCGAC ATCGCGTTGA ATCCTT.TGG TCACGAATGA CGTATGCACC GGAATGTTTC .GAAATCTTC TTTCTTTTTT TCTTTTTTTC TTTTTTCCGA GCGAAAAG.. ....TTTCAA TTCGCTGCAA
 Drosophila TATTGCCCGT CAGTTGGTGG AGAATGCTCC GGGAAATCGG TTCAATGTGA TCCTGGGCGG AGGAATGTCG CCCATGGGCA TCCTGAATGC CTCCGAGGTG AAGACTACTA TTTTCGAAGG ACCCACGGAA ACAATTTGCA CCCGCGGTGA
     Bombyx CATCGCTCAC CAGCTGATCA AGATGGCGCC AGGAAACAAA TTTAAAGTGA TCTTTGGCGG AGGAAGACGA GAATTTTTAC CGACAACCCA AGTCGATG.. .......... ...AAGAGGG CACCCGAG.. .......GTC TAAGAACAGA
     Gallus CATCGCCCGG CAGCTGGT.. GGACAACATC CCTGA.CA.. .TCGAGGTGA TCCTGGGGGG TGGGCGCAAG TACATGTTCC CCAAGAACAC CAGCGATGTG GAGTACCCGC AGGAGGAGCG GCACCGCG.. .......GCA CCCGCCTGGA
     Bovine CATCGCCTAC CAGCTCAT.. GTACAACATT AAGGA.CA.. .TCGAGGTGA TCATGGGCGG CGGCCGGAAG TACATGTTCC CCAAGAACAG AACCGATGTG GAGTATGAGC TGGATGAGAA GGCCAGAG.. .......GCA CGAGGCTGGA
       Homo CATCGCCTAC CAGCTCAT.. GCATAACATC AGGGA.CA.. .TTGACGTGA TCATGGGGGG TGGCCGGAAA TACATGTACC CCAAGAATAA AACTGATGTG GAGTATGAGA GTGACGAGAA AGCCAGGG.. .......GCA CGAGGCTGGA
  Consensus cAtcgc.ca. cAg.T..t.a ..Aat.Cg.C a.gga.t.ga .Tc.atgTGa TCatggg.Gg .Gga.g.ac. g.aaTgtt.c .gaaaact.c .ttCgatgt. ...t.t..tc tttt.ga.ga gccca.aG.. ....tttg.a t.cGc.g.gA

            451                                                500                                                550                                                600
       Apis CGTCGAGTGG AAGGAGGTCG CATTGAC... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
 Drosophila TAACAGAAAC CTTCCTGCCG AGTGGCTGGC CCATCACG.. CCAACGACAC AGTTCCTCCA GCATTGGTAC ATAACCGTAA GGATCTGCTT AATGTGAATG TCAAGAAG.G TGGACCATTT GATGGGCCTG TTCCGAAACA ATCACATTAC
     Bombyx CGGCCGCAAT CTGATCGAAG AATGGCAGAA CGATAAGGAG TCGCAGAAAG TGAGCTACAA GTATCTGTG. .GAATCGACA GGAACTTTTG AAACTGGGTT CGTCTCCGCC CG.ACTACCT GCTCGGACTG TTCGAGGGCA GTCACTTGCA
     Gallus CGGCAAGGAC CTGGTGCAGG CGTGGCACGA CACTAAG... CCTGCGGGGA AGGTCGCCAA GTACGTGTG. .GCACCGGAG GGAGCTGCTG GCGCTGAACG T.CAGCCGTG TGGACTTCCT GCTGGGTCTC TTTGAGCCGG GGGATATGGT
     Bovine CGGCCTGAAC CTCATCGACA TCTGGAAGAG CTTCAAA... CCGAAACACA AGCACTCTCA CTATGTCTG. .GAACCGCAC TGATCTCCTG GCCCTTGACC C.CCACAGCG TGGACTACCT CTTGGGTCTC TTTGAGCCGG GGGACATGCA
       Homo CGGCCTGGAC CTCGTTGACA CCTGGAAGAG CTTCAAA... CCGAGATACA AGCACTCCCA CTTCATCTG. .GAACCGCAC GGAACTCCTG ACCCTTGACC C.CCACAATG TGGACTACCT ATTGGGTCTC TTCGAGCCAG GGGACATGCA
  Consensus cg.C..g.a. ctgg..G.cg caTgGa.g.. c....a.... .c........ .g..c..c.a ..a....t.. ..aa.cg... gga.ct..t. ....t..... .......g.. .g.ac.a..t ..t.gg.ct. tt........ ...ac.t...

            601                                                650                                                700                         730
       Apis .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
 Drosophila GTACTCCATA GCCAGGGAGG CGGGA...GA GCCTTCCCTG CAAGAGATGA CGGAGACGGC CTTGGGAATC TTGGAAAGGG ACGATGAGTC CAACGGCTTT GTGCTCCTGG TGGAAGGAGG TCGCATTGAC
     Bombyx GTACCATCTC GAGGGAGATG AGAGCACGGA GCCAACCCTC GCCGAGCTAA CAGACGTTGC CATCCGCGTG CTGAGTCGCA ACG......A GCGCGGTTTC TTCTTGTTCG TGGAGGGAGG GCGCATCGAC
     Gallus GTATGAGCTG GACAGGAACA ACGAGACCGA CCCGTCGCTC AGCGAGATGG TGGCTGTGGC CATAAGGATG CTGCAGAAAA ACC......C GCGGGGCTTC TTCCTGCTGG TGGAAGGAGG CCGCATCGAC
     Bovine GTACGAACTC AACAGGAACA ATGCGACTGA CCCTTCACTC TCTGAGATGG TAGAGATGGC CATCAGGATC CTGAACAAGA ACC......C CAAAGGCTTC TTCCTGCTGG TGGAAGGGGG CAGGATTGAC
       Homo GTACGAGCTG AACAGGAACA ACGTGACGGA CCCGTCACTC TCCGAGATGG TGGTGGTGGC CATCCAGATC CTGCGGAAGA ACC......C CAAAGGCTTC TTCTTGCTGG TGGAAGGAGG CAGAATTGAC
  Consensus gtac....t. ....g..a.. ........ga .cc..c.ct. ...gag.t.. ..g.....gc c.t..g..t. .tg....... ac........ ....gg.tt. .t..t..t.g tgga.ggagg ..g.at.gac
```

Figure 77. Comparison partial nucleotide sequence of *AP* in *A. mellifera*, *D. melanogaster*, *B. mori*, *G. gallus*, Bovine, and *Homo* are compared by Mutalin program at http://prodes.toulouse.inra.fr/multalin/result/1043178322.msf.

```
               1                                              50                                                 100
        Apis   .......... .......... .......... ......VPDS AGTATLGKGA HISGKARSWL KDYELEAVKI MVDDPTIYRR SNKIYREFLP RS.SFSFNIP
  Drosophila   VPDSAGTATA IFSGSKTHYG AIGMDATRSK .....KNGQQ GRVQSVMEWA QKEGKRTGVV TTTRITHATP AATYATSTTG TGSATRKCPR NRWAFMSILP
     Bombyx    VPDSSCTATA YLCGVKANQG TPGVTAAVPR HDCEASTDVT KRVQSIAEWA LADGRDVGIV TTTRITHASP AGTFAKVANR NWENDNDVKQ EGHDVNRCPD
     Gallus    VPDSAGTATA YLCGVKANEG TVGVSAGVTR DRC..NTTKG QEVTSILRWA KDEGKAVGIV TTTRVTHATP SAAYAHSANR DWYSDGEMPL DALE.GGCKD
     Bovine    VPDSAGTATA YLCGVKANEG TVGVSAATQR SQC..NTTQG NEVTSILRWA KDAGKSVGIV TTTRVNHATP SASYAHSADR DWYSDNEMPP EALS.QGCKD
       Homo    VPDSAGTATA YLCGVKANEG TVGVSAATER SRC..NTTQG NEVTSILRWA KDAGKSVGIV TTTRVNHATP SAAYAHSADR DWYSDNEMPP EALS.QGCKD
  Consensus    vpdsagtata ...g.k...g ..g..a.... .......... ..v.s...wA ...Gk..g.v tttr.thatp .a.yats..r .....re.p. ....f....p

               101                                            150                                                200
        Apis   CARGVGGIEG GMEGRGVASK LTTDRIYKIR HRVESFGHER MHRNVSKSSF FFSFFLFSER KVSIRCNVEW KEVALT.... .......... ..........
  Drosophila   VSWWRMLREI GSMSWAEECR PWASMPPRRL LFSKDPRKQF APAVITETF. LPSGWPITPT TQFLQHWYIT VRICLMMSRR WTIWACSETI TLRTPPGRRE
     Bombyx    IAHQLIKMAP GNKFKVIFGG GRREFLPTTQ VD.....EEG TRGLRTDGRN LIEEWQNDKE SQKVSYKYLW NRQELLKLGS SPP...DYLL GLFEGSHLQY
     Gallus    IARQLVDNIP D..IEVILGG GRKYMFPKNT SDVEYPQEER HRGTRLDGKD LVQAWHDTKP AGKVA.KYVW HRRELLALNV SRV...DFLL GLFEPGDMVY
     Bovine    IAYQLMYNIK D..IEVIMGG GRKYMFPKNR TDVEYELDEK ARGTRLDGLN LIDIWKSFKP KHKHS.HYVW NRTDLLALDP HSV...DYLL GLFEPGDMQY
       Homo    IAYQLMHNIR D..IDVIMGG GRKYMYPKNK TDVEYESDEK ARGTRLDGLD LVDTWKSFKP RYKHS.HFIW NRTELLTLDP HNV...DYLL GLFEPGDMQY
  Consensus    .a......e. g......... ....m.pk.. ..ve....#. .......... l.s.w..... ......y.w .r..L..... .......... .l..p.....

               201                                  240
        Apis   .......... .......... .......... ..........
  Drosophila   SLPCKRRRRP WESWKGTMSP TALCSWWKEV ALT.......
     Bombyx    HLEGDESTEP TLAELTDVAI RVLSRNERGF FLFVEGGRID
     Gallus    ELDRNNETDP SLSEMVAVAI RMLQKNPRGF FLLVEGGRID
     Bovine    ELNRNNATDP SLSEMVEMAI RILNKNPKGF FLLVEGGRID
       Homo    ELNRNNVTDP SLSEMVVVAI QILRKNPKGF FLLVEGGRID
  Consensus    .l......p ..s....... ..l....... .l........
```

Figure 78. sequence alignment of partial amino acid of *AP* in *A. mellifera*, *D. melanogaster*, *B. mori*, *G. gallus*, Bovine, and *Homo* are converted by EMBOSS-Transeq at http://www.ebi.ac.uk/servicestmp/ and were compared by Mutalin program at http://prodes.toulouse.inra.fr/multalin/result

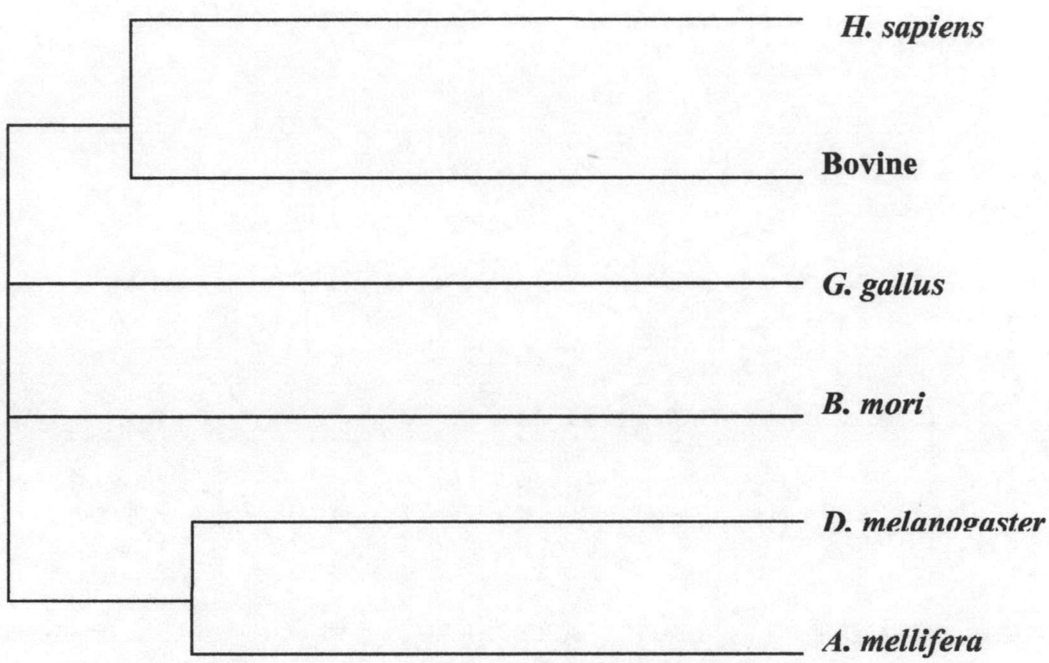


Figure 79. Phylogenetic tree of non specific *AP* in *A. mellifera*, *D. melanogaster*, *B. mori*, *G. gallis*, *H. sapiens*, and Bovine were compared by Clustal W (1.82) Multiple Sequence Alignments of partial nucleotide and amino acid sequence at http://www.ebi.ac.uk/clustalw/.

4. AP in crude extraction of *A. mellifera*

4.1 The proper concentration of PNPP and incubation time to detect AP in crude extract

The proper concentration of PNPP was more than 0.15 μM (Figure 80A). Michaelis-Menten plot showed the maximum activity at 0.15 μM and would be stable activity until 0.15 μM to 1.2 μM. For this research, the PNPP concentration of 0.30 μM was used in all experiments because it was more enough to assay AP activity in reaction (Figure 80A). According to the varied incubation time, it showed that the time from 45- 90 min was the most properly to assay because of the colormetrically detection of reaction. The Michaelis-Menten plot showed high activity when incubation time increases (Figure 80A and 80B).

4.2 Optimum pH in crude extract

Tris-HCl pH 6.5 to 9.5 was used as buffer to quantitate pH of reaction. The highest AP activity was shown at pH 7.0. After that, the activity would decrease. The optimum pH was a final pH of reaction because of the effect of substrate buffer. From the above reason, the final pH of reaction was determined again. So, the optimum pH of the AP in *A. mellifera* was at the final reaction (Figure 81).

4.3 Optimum temperature in crude extract

The mixture was incubated at 35, 40, 45, 50, 55, 60, and, 70°C, respectively. The temperature started from 35°C to 50°C. It showed that the AP activity increased during this duration and became the highest at 50°C. The AP activity decreased if the temperature is higher than 50°C. The optimum temperature of the AP activity in crude extract was at 50°C (Figure 82).

4.4 Amount of total protein in crude extract

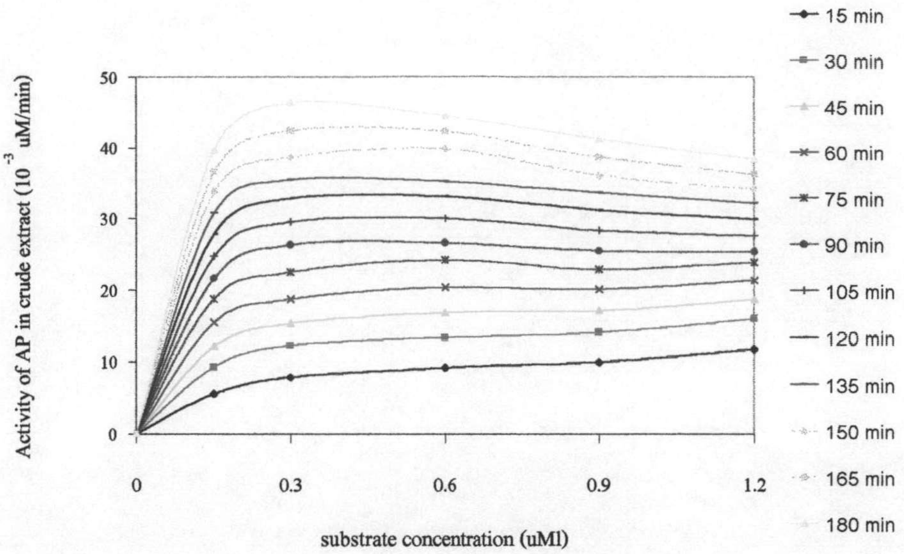
Amount of total protein in crude extract (1 mg sample) from three colonies of *A. mellifera* in each stage of development were shown in table 3. Means of amount of total protein were different in each stage of development and in each colony. The lowest amount of protein was from egg at 48 h (1^{st}) of developmental stage (Table 4). At larva stage (2^{nd} to 4^{th}), the amount of protein is fluctuated. The amount of total protein was dropped at prepupa stage (6^{th}) and it was high at pupa stages (7^{th} to 9^{th}). At emerging adult (10^{th}), the amount was dropped again (Figure 82). From this result, at 1^{st}, 6^{th}, and 10^{th} were highly morphogenesis in development. Amount of protein would be to develop tissue or organ so that it was lower amount of protein than other stages.

4.5 AP activity in crude extract

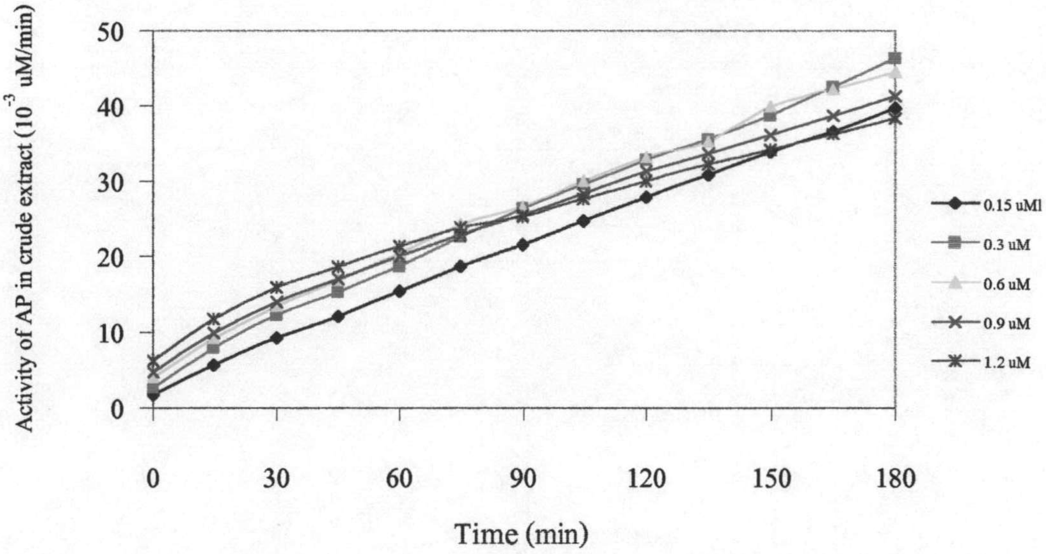
The AP activity in crude extract was determined by hydrolysis. The activity of AP was calculated from the standard graph of PNPP by the absorbance comparison (Figure 85). Then, the specific activity in each developmental stage was calculated. Specific activities from samples of all stages of development were significantly different within 3 colonies (Table 5). The maximum specific activity showed in larva at 144 h (4^{th}) of development. The specific activities in all pupa stages were lower than in larva stages (Figure 86).

Time of development (h)	Means of amount of total protein (mg) in 1 mg sample	Means of specific activity of AP($\times 10^{-6}$ U/mg protein) in 1 mg sample
Egg (1^{st})	543.98 ± 95.91	16.92 ± 5.90
48 (2^{nd})	4,367.28 ± 1,065.99	36.83 ± 10.09
96 (3^{rd})	4,876.54 ± 459.91	84.68 ± 2.65
144 (4^{th})	3,978.01 ± 1,134.76	119.77 ± 24.25
192 (5^{th})	4,820.60 ± 448.53	49.03 ± 11.51
240 (6^{th})	3,472.22 ± 512.38	42.38 ± 6.92
288 (7^{th})	4,560.19 ± 355.03	43.76 ± 7.87
336 (8^{th})	4,833.33 ± 212.19	42.09 ± 1.91
384 (9^{th})	4,696.76 ± 178.49	75.27 ± 7.84
432 (10^{th})	3,390.05 ± 426.82	100.20 ± 1.86

Table 5. Means of amount of total protein (mg) and specific activity of AP (U/mg protein) in 0.1 mg in crude extract at different developmental stages of *A. mellifera* from 3 colonies.



A



B

Figure 80. The relationship of incubation time and concentration of substrate (PNPP) with AP activity in crude extract. The incubation time was 45-90 min (A). Concentration of PNPP used to determine AP activity in crude extract was 0.3 μM (B).

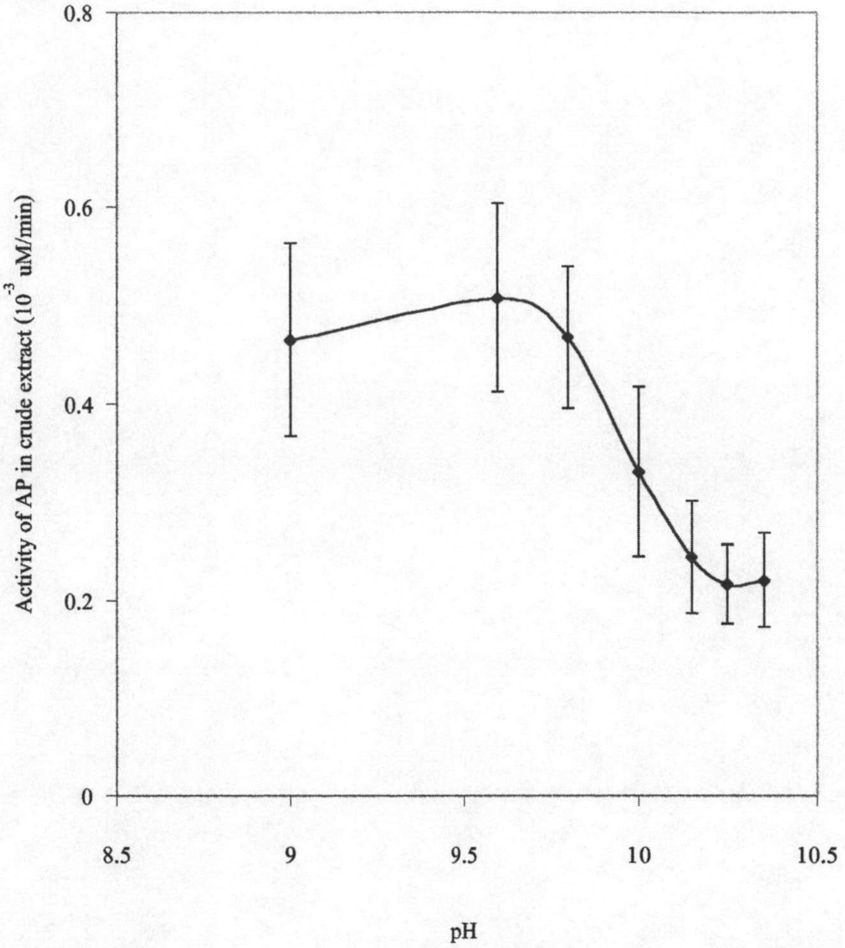


Figure 81. The optimum pH of AP in crude extract of *A. mellifera* was determined three times. Means of AP activity was measured in Tris-HCl at pH range 6.5-9.5. The final pH of all reactions was 9.0-10.35. The optimum pH was in 0.1 M Tris-HCl at pH 7.0 or in final reaction at pH 9.6.

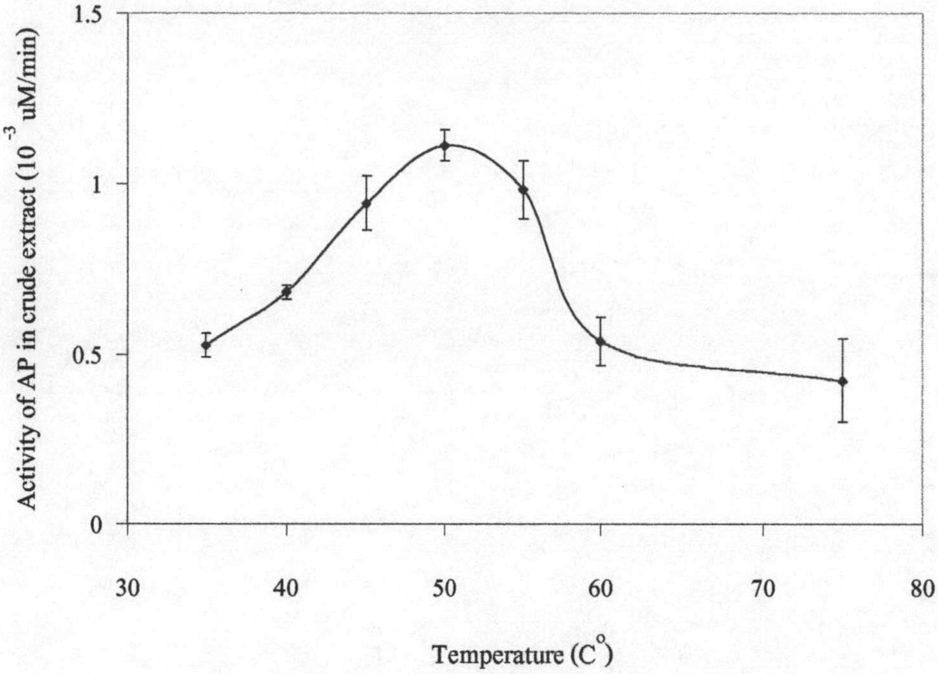


Figure 82. The optimum temperature of AP in crude extract of *A. mellifera* was determined three times. Means of AP activity was measured at 35, 40, 45, 50, 55, 60, and, 70°C, respectively. The optimum temperature of the AP was 50°C.

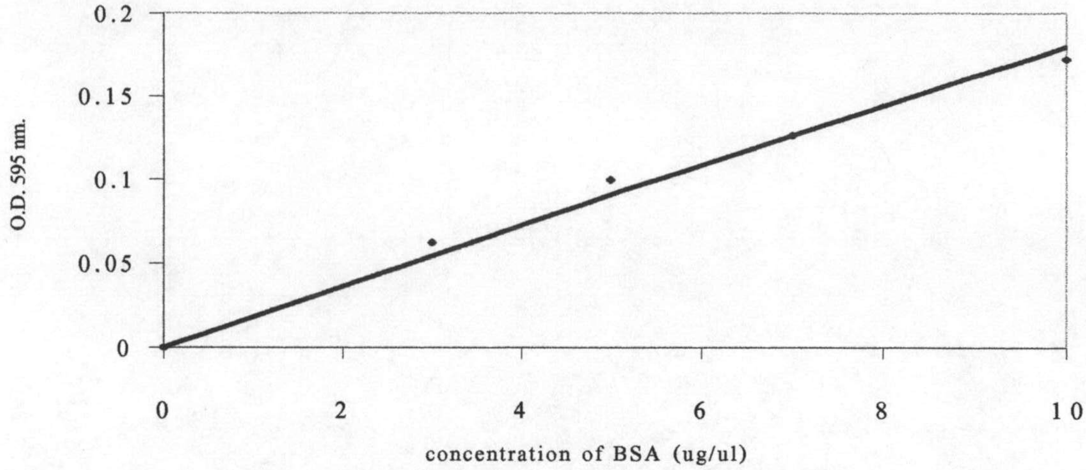


Figure 83. Graph of standard BSA was related to the amount of total protein and the standard BSA protein at 595 nm. The slope of graph was used to calculate an unknown concentration of total protein in crude extract.

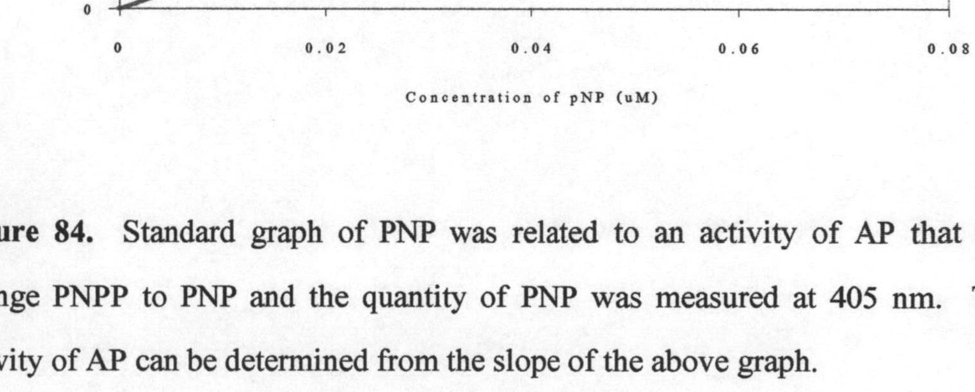


Figure 84. Standard graph of PNP was related to an activity of AP that can change PNPP to PNP and the quantity of PNP was measured at 405 nm. The activity of AP can be determined from the slope of the above graph.

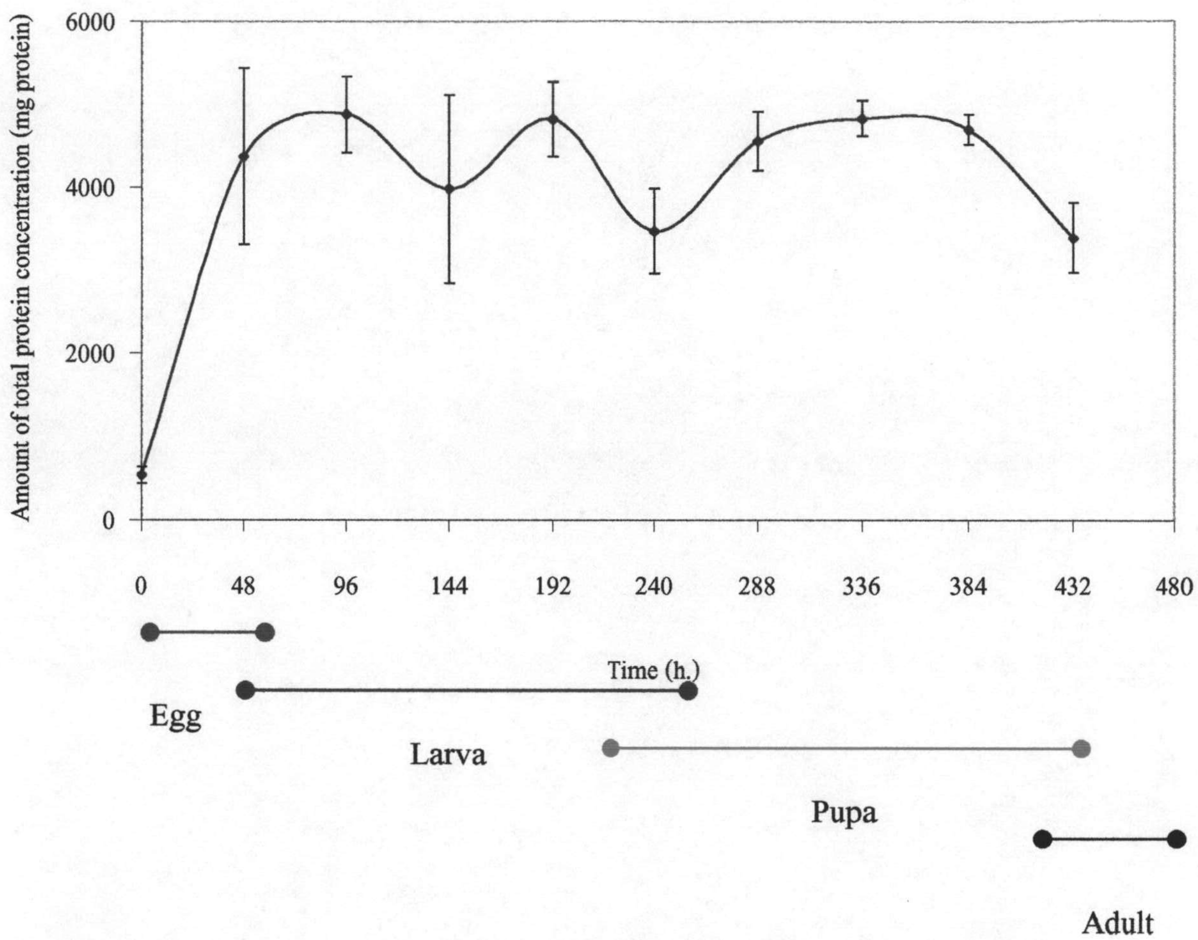


Figure 85. Amount of total protein was determined from three colonies. All of colonies showed resemble trend of amount of total protein in each developmental stage. Mean of amount of total protein in developmental stage was fluctuated during 48 h egg stage (1^{st}), prepupa (6^{th}), and emerging adult (10^{th}). The amount of total protein was lower than another periods.

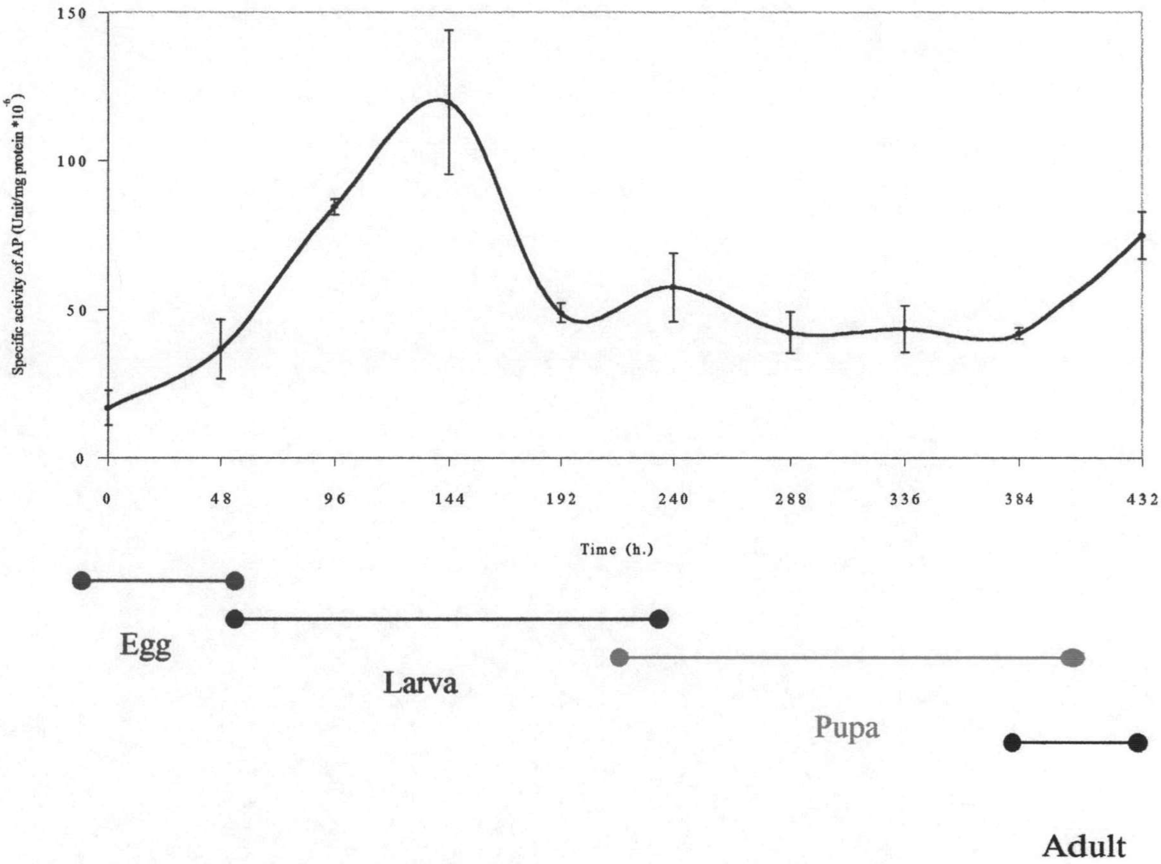


Figure 86. Specific activity was determined from three colonies. All of colonies showed resemble trend of specific activity in each developmental stage. The specific activity was the highest in samples at 144 h (4^{th}). Also, specific activity in larva at 96-144 h stage was higher than pupa at 288-384 h stage. Later, the specific activity was getting high again at 432 h emerging adult stage (10^{th}).

5. Detection of AP activity in each developmental stages of *A. mellifera* by polyacrylamide gel electrophoresis (PAGE)

5.1 Native Polyacrylamide Gel Electrophoresis (Native PAGE)

The protein was stained with NBT/BCIP to determine the AP activity in gel by Native PAGE. The positive band of activity was visible colormetrically on a gel at 3 h. If the staining period was longer, the darker color will be developed. The activity of AP was shown at 2^{nd}, 3^{rd}, 4^{th}, and 10^{th} stages. In addition, the highest activity was present at 2^{nd}, 3^{rd}, and 4^{th} stages (Figure 87A).

5.2 SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

For the AP activity staining, SDS, which can denature protein, was concentrated to be 0.1% (w/v). This indicated that only 0.1% (w/v) SDS can not denature the AP protein completely (Verhaert *et al.*, 1990). The positive band of AP activity was shown at 2^{nd}, 3^{rd}, and 4^{th} after stained in NBT/BCIP. Incubation of the crude extract at 30°C and 50°C was not able to denature the AP protein. In contrast, a little AP activity was shown from the crude extract incubated at 70°C for 5 min. The protein was completely denatured at 100°C for 5 min. The AP activity of *A. mellifera* was tolerant to 0.1 % (w/v) SDS and heating. That is similar to the AP in *P. americana* (Verhaert *et al.*, 1990).

For coomassie blue staining, the sample of SDS PAGE was denatured with 0.1% (w/v) SDS and heating 100 °C for 5 min. It presented that the mass weight of AP is about 150 kDa (Figure 87B and 88B).

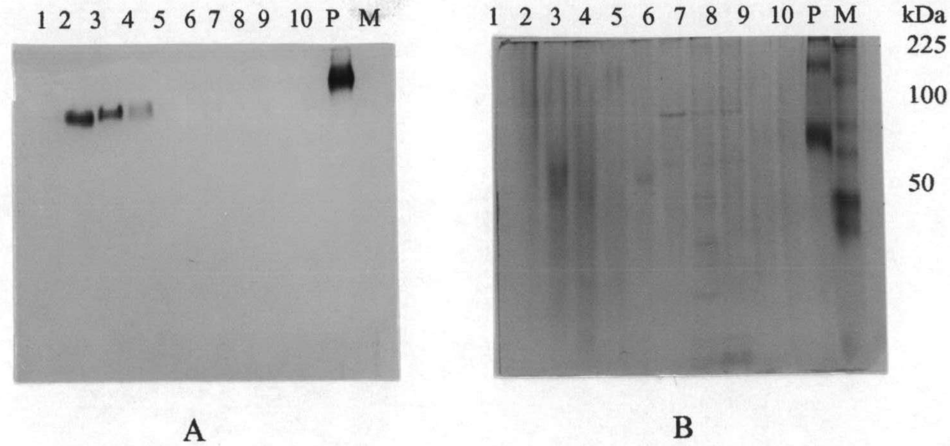


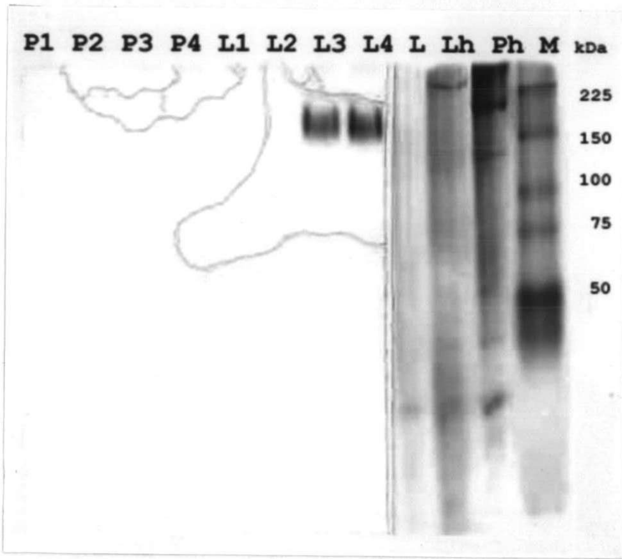
Figure 87. Protein pattern was shown by activity staining (Native-PAGE) (A) and coomassie blue staining (SDS-PAGE) (B). Calf mucosa intestinal-AP was used as positive control (A and B). Native PAGE showed high AP activity at 2nd, 3rd, 4th, but a little at 5th and 10th stages. This native gel was stained by 350 μg/ml NBT and 175 μg/ml BCIP in 0.1 M Diethanolamine containing 5 mM $MgCl_2$ at pH 10.5 and Tris-HCl at pH 7.0 with the ratio of 1:5. The color detection time was longer than 3 h. The AP activity changes the color mixture of NBT and BCIP to be purple (A).

M represents Broad Range Protein Molecular Weight Markers (kDa)

P represents Positive control calf mucosa intestinal-AP

1, 2, 3,4, 5, 6, 7, 8, 9 and 10 represent 20 μg protein in crude extract of 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, and 10th stages

Negative control was not shown



A B

Figure 88. Protein pattern was shown by activity staining (Native-PAGE) (A). Samples were heated and incubate at 30°C, 50°C, 70°C, and 100°C for 5 min. The activity was higher activity at 30°C and 50°C in larva stage than in pupa stage. The mass weight of AP is about 150 kDa that was shown by SDS PAGE (B).

P1, P2,	represent	crude extract of 7th pupa stages were heated at
P3, and P4		100°C, 70°C, 50°C, and 30°C, respectively.
L1, L2,	represent	crude extract 4th larva stages were heated at
L3, and L4		100°C, 70°C, 50°C, and 30°C, respectively.
L and Lh	represent	crude extract 4th larva stages were heated at 30°C
		and at 100°C after that stained by Coomassie blue
Ph	represents	crude extract 7th pupa stages were heated at
		30°C and Coomassie blue staining
M	represents	Broad Range Protein Molecular Weight Markers (kDa)