CHAPTER II

LITERATURE REVIEWS

Biology of *A. mellifera*

Honeybees in genus *Apis* can build single or multiple colonies. *A. mellifera* can also build multiple colonies in a dark area, such as the hole of tree (Figure 1A), underneath of roof or a box. It provides the data that this species can be maintained in a hive (Figure 1B). Colonies have been developed in order to survive in cold winter by forming a cluster within the shelter of the nest cavity (Michener, 1974; Crane, 1999). They are social insects divided into three levels. A queen and workers are female containing two sets of chromosomes. In one single colony, there can be only one queen. The reproductive system of queen is well developed in order that it can lay eggs in cells. It releases queen pheromone to control workers in its colony. In a colony, almost honeybee population are workers. Also, they do many tasks in the colony (Figure 2). The task is processed by different ages of honeybee. For example, nurse bees feed larva while guard bees protects the colony. Also, forager bees forage pollen and nectar. Drone is male which contains only one set of chromosomes. The duty of drone is only to fertilize to queen. After that, it will die shortly (Michener, 1974; Wongsiri, 1989).

Classification of *A. mellifera*

A. mellifera means the honey-bearing bee. Its name is firstly given by Linneaus in 1758. Its common name is "Western honeybee". It is indigenous only in Europe and Africa. Later, this species has of course been introduced from Europe into almost every country in the world (Ruttner, 1988; Dade, 1994). *A. mellifera* is classified as follow:

Kingdom Animalia

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Superfamily Apoidea

Family Apidae

Subfamily Apinae

Genus *Apis*

Species *Apis mellifera*

Development of *A. mellifera*

The development of *A. mellifera* is similar to that of other holometabolous insect (complete metamorphosis). Each individual passes through egg, larva, pupa, and adult stages. The larva and adult are in the feeding stages. While the larva is in the growing stage, the pupa is in the stage of differentiation and formation of adult parts (Table 1, Figure 3). During metamorphosis, the extensive degradation of several larval tissues takes place. This synchronized process in honeybee larvae is balanced by two hormones: ecdysteroid and juvenile hormone. Ecdysteroid is responsible for induction of metamorphosis and causes the degradation of larval tissues. Juvenile hormone for qualitative changes during metamorphosis (Rachinsky *et al.*, 1990).

1. Egg stage

An egg of *A. mellifera* is in small size. The eggs are slightly larger than 1.5 mm. They are attached to the bottom of cell in a horizontal posture and glued to the floor by gummy substance. One egg weighs about 0.13 mg.

2. Larva stage

The larva is a whitish and legless grub. It is very soft, delicate and curled. They can be either fed by the stored food in mass provisioning forms or fed at intervals by adult bees in progressive feeders. It has little ability to move. Its sensory and protective features are greatly reduced when they are compared to

those in primitive Hymenoptera. Larva usually grows rapidly. The larva of bees spends most of its time in shelter. It is protected and fed within the nest, and is almost totally inactive. It is also blind and the mouthpart is such suitable to suck up semi-fluid food. During the larval phase development, the increase of size is virtually limited (Figure 4A).

Segmentation is conspicuous throughout the larval body, except in the head, where the consolidation of the foremost segments was already complete before hatching. The internal organs also show their segmental origin very clearly. After hatching, the larva grows very quickly (Figure 5A and 5B). The stages of growth and the moults are called stadia and ecdyses, respectively. The form taken by the larva during a stadium is an instar. The moulting process consists of the softening and dissolving of the inner layer of the cuticle. The epidermis then secretes new cuticle layers.

After the 4th moult, the larva makes the most growth. About two days after this moult, the larva receives its last meal, and its cell is sealed. The larva changes its position and stretches itself out along the cell on its back with its head towards cap of the cell. During this stage, the larva goes through the prepupal instar. This complete change of form is called metamorphosis (Figure 4B and 5B).

3. Pupa stage

The pupa is relatively delicate and whitish. Within this stage, it forms to be an adult insect except the appendages are still folded and the wing is still small. The 5th moult occurs and the pupa will be revealed with all the external features of the adult insect except wings. The pupa is soft and white. During moulting, the lining of the foregut, hindgut, tracheae, glands, and larva skin are cast off. During the eight day of this instar, the pupa gradually assumes color. Beginning with eyes, they become pink, purple, and then brown (Figure 4C and 5B).

4. Adult stage

All of adult honeybees are in active stage. They do many tasks in a colony. The body is divided into three major tagmata (head, thorax, and abdomen). The first abdomenal segment is fused with and in fact fully incorporated into the thorax. This segment is called propodium. All internal organs in adult such as alimentary, circulation, nervous, muscle system, etc. function completely (Figure 6).

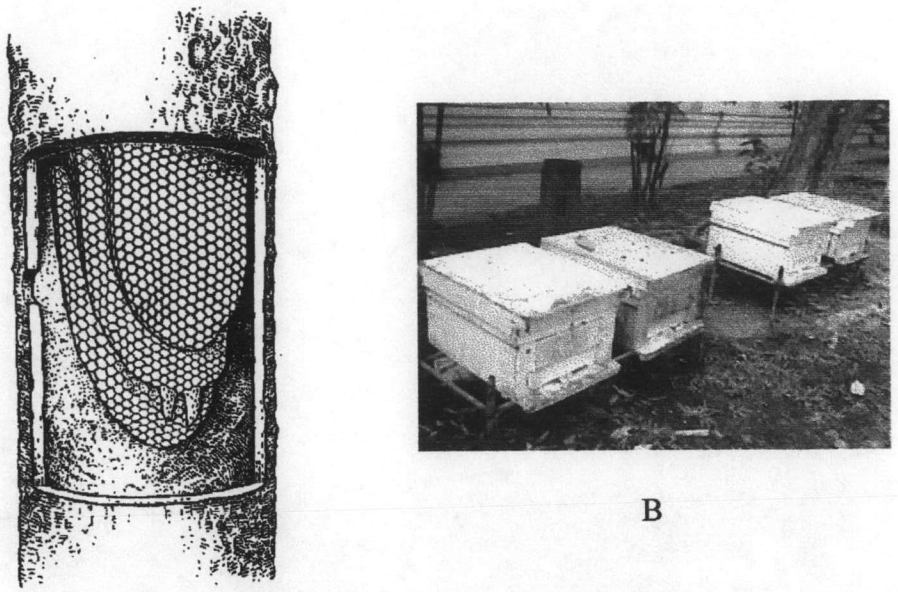
The mouthpart is a combination of chewing or biting and sucking types. Labrum and mandibles are attributed to the former while maxillae and labium are attributd to the latter. The mandibles are used in nest making. The maxillae and labium together form a proboscis is used to take up liquid such as nectar, honey, and water. The proboscis involves in a formation of a tube around a glossa.

The leg structures used for carrying pollen consist of scopal hair at various locations. Also, the hair arranges underneath of the abdomen or at hind legs. A corbicular in a pollen basket on the hind tibia is for transportation of resins and sometimes for other nest making materials.

Most of female honeybee can sting. The sting is a modified ovipositor in which the valvulae of the eight segment are fused and expanded to form a bulb for containing poison (Figure 6) (Michener, 1974; Dade, 1994).

Weeks	Days	Queen	Worker	Drone
1	1	Egg laying	Egg laying	Egg laying
	2			
	3			
	4	Hatch	Hatch	Hatch
	5			
	6		Diet changes	Diet changes
	7			
2	8			
	9	Sealing	Sealing	
	10			
	11			Sealing
	12	5th moult		
	13			
	14		5th moult	
3	15			
	16	Emerges		
	17			5th moult
	18			
	19			
	20			
	21	Mature		
4	22		Emerge	
	23	Mate		
	24			
	25			Emerge
	26			

Table 1. Life-histories of *A. mellifera* (Dade, 1994).



A B

Figure 1. Multicombs of *A. mellifera* colony are located in a hole of tree (A) (Michener, 1974) and in hives (B).

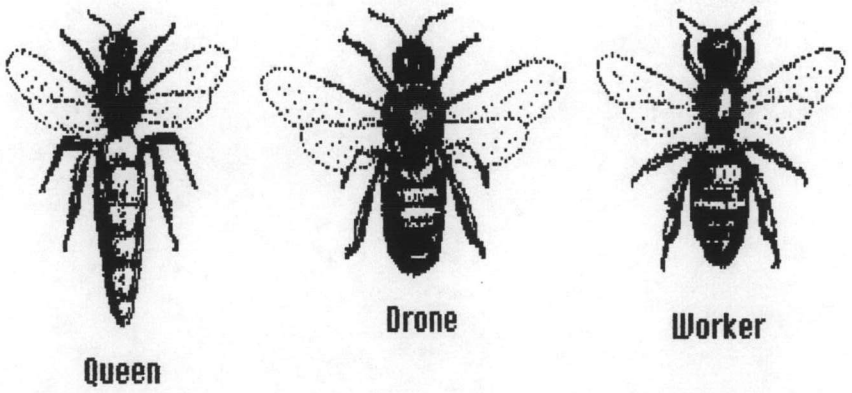


Figure 2. In one colony, honeybees can be divided into three levels those are a queen, workers, and drones.

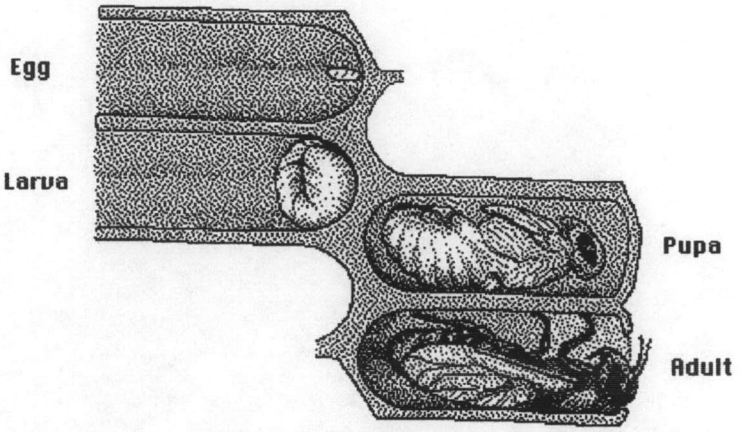


Figure 3. The development of *A. mellifera* is holometabolous. It develops into 4 stages of egg, larva, pupa, and adult.

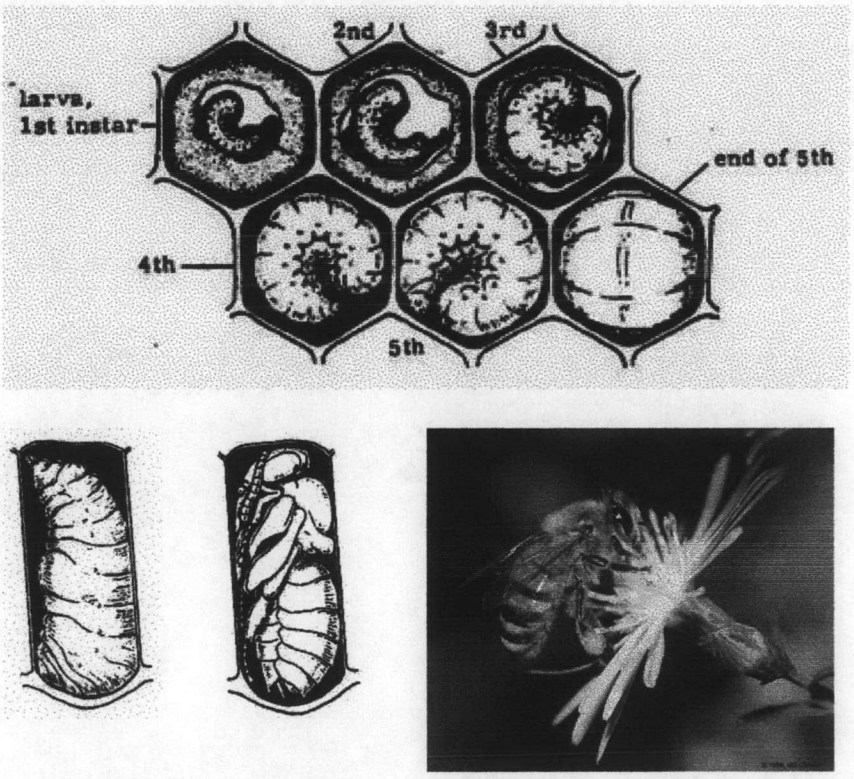
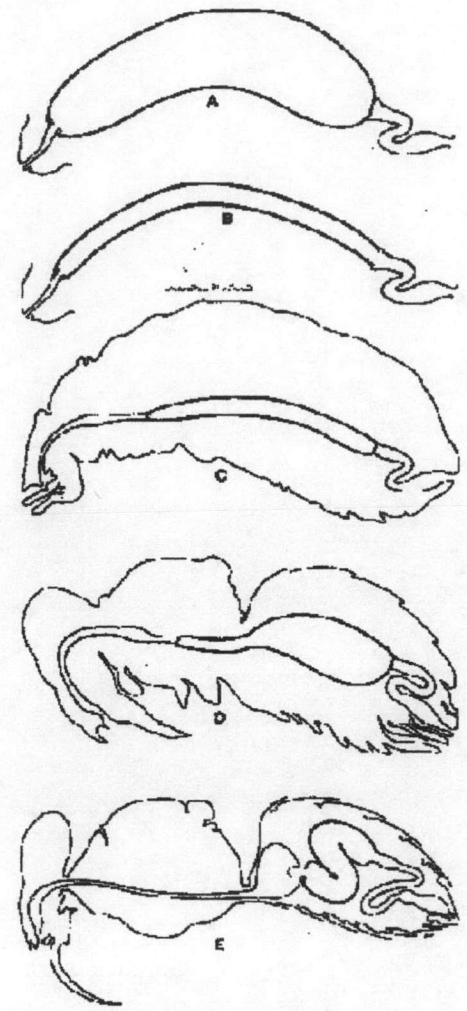


Figure 4. Metamorphosis in larva stages (A), in prepupa stage (B), in pupa stage (C), and in adult stage of development (D) (Dade, 1990).



A

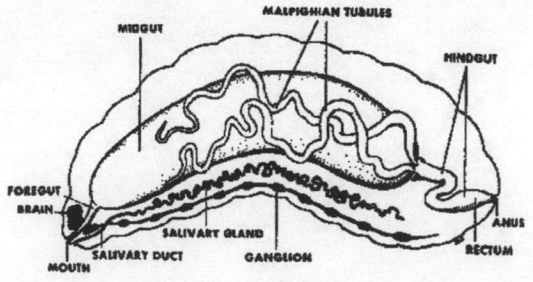


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Figure 5. Internal organ of larva stages, midgut, malpighian tubule, gland, and others (A). Alteration of gut in honeybee development from larva to adult (B) (Michener, 1974).

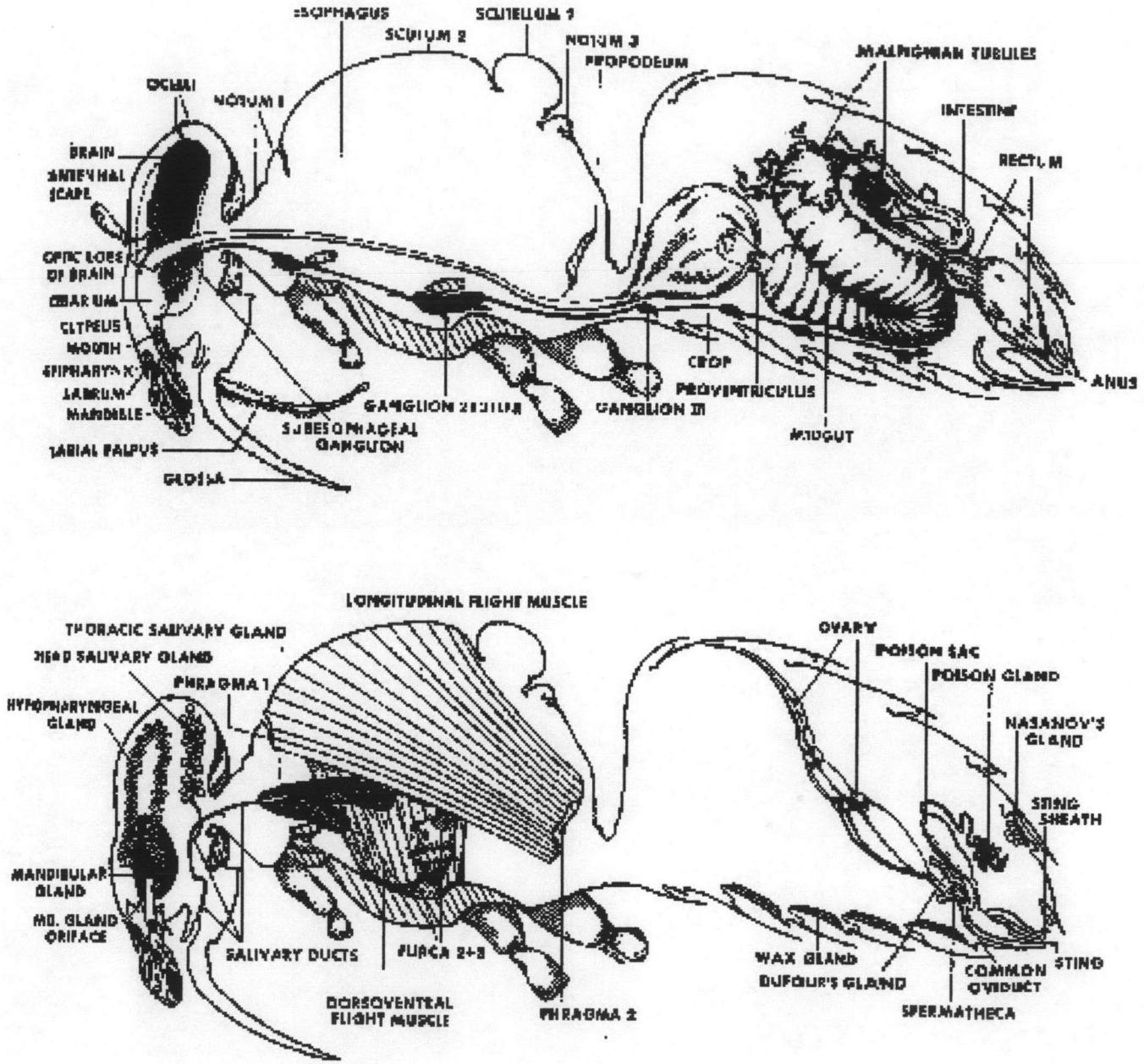


Figure 6. Anatomy of internal organs of adult bees (Michener, 1974).

Alkaline phosphatase (AP)

AP is a widely distributed enzyme in several organisms and their properties have been extensively studied. About the nomenclature of AP, it is an orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1. (McComb *et al.*, 1979). Most of AP is a metalloenzyme which is tightly bound to metal ions such as ferrus (II), (III) ions, copper (I) ion, zinc (II) ions, manganis (II) ions or cobalt (II) ions, as cofactors. It can hydrolyse phosphate esters when the optimum pH is high. The enzyme requires the presence of metal ions for catalytic activity. Metal ions are more effective catalysts than protons because they can be present in high concentrations at neutral pH. The enzyme hydrolyses phosphate mono esters as follow:

$$R\text{-}O\text{-}PO_4 + H_2O \xrightarrow{AP} R\text{-}OH + PO_4^{3-}$$

(Voet and Voet, 1995).

AP is found in most species from bacteria to human. In *Escherichia coli*, AP is found in the periplasmic space. In yeast, AP is found in lysosome-like vacuoles. In mammals, it is a glycoprotein attached to the membrane by a glycosylphosphatidylinositol anchor (McComb *et al.*, 1979).

The optimum pH of AP is between 8.0-10.5. In addition, the activity and thermal stability of the enzyme depend on proper temperature (Halford, 1971). AP is very useful in enzyme-immunoassays (EIA), in nucleic acid based diagnostic assays, and in molecular biology for dephosphorylation of nucleic acid (Verhaert *et al.*, 1990).

AP hydrolyzes phosphate esters of primary and secondary alcohol, cyclic aliphatic alcohols, sugar alcohols, phenols, and amines. It also hydrolyzes inorganic pyrophosphate and 5'-terminal phosphates of single and double-stranded DNA or RNA. The hydrolysis rate and substrate affinity of AP depend on a complex interrelation between the enzyme purity, enzyme concentration, buffer composition, ionic environment (ionic strength), and pH. Specific use of AP

requires careful and empirical optimization of the reaction condition (Gomori, 1949; Dikow *et al.*, 1990; Thompson *et al.*, 1991).

Some of AP is a dimer. Each contains metal molecules such as zinc (II) ions, magnesium (II) ions or manganis (II) ions. The first part of dimer is tightly bound to either mentioned ion which is necessary for structural stability while the other part is loosely bound to ions which is required for catalytic activity (Halford, 1971). Then, it can say that the activators of AP are divalent metal ions. Furthermore, amino alcohols (2-amino-2-methyl-1-propanol and diethanolamine) and Tris-HCl is reported as activators as well. The optimum activity of AP depends on the concentration of magnesium (II) ions and zinc (II) ions in the reaction mixture (Halford, 1971; Brown *et al.*, 1974; Anderson *et al.*, 1975).

The substrate specificity is important to study an enzyme activity. In histochemical specificity, the activity of phosphatase in non-specific organs of human was studied. Nineteen different substrates were tested for the activity but there was only one substrate that shows the substrate specificity (Gomori, 1949).

At present, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) together with a number of different chromogens are used in various histological and molecular biology techniques. AP can hydrolyse indolyl phosphate and can produce a blue color precipitating at the site of enzymatic activity. Mostly, nitroblue tetrazolium (NBT) is commonly used as an electron-transfer agent and a co-precipitant to BCIP. They will provide dark blue product (Figure 7). BCIP and NBT is widely used to study the activity of AP (Verhaert *et al.*, 1990).

About cytochemistry, para-nitrophenol phosphate (PNPP) is commonly assayed by spectrophotometry. Phosphatase enzyme converts PNPP to para-nitrophenol (PNP) (Figure 8). In basic solution, it shows highly yellow and absorbs light at wavelength of 400-410 nm (Thompson *et al.*, 1991).

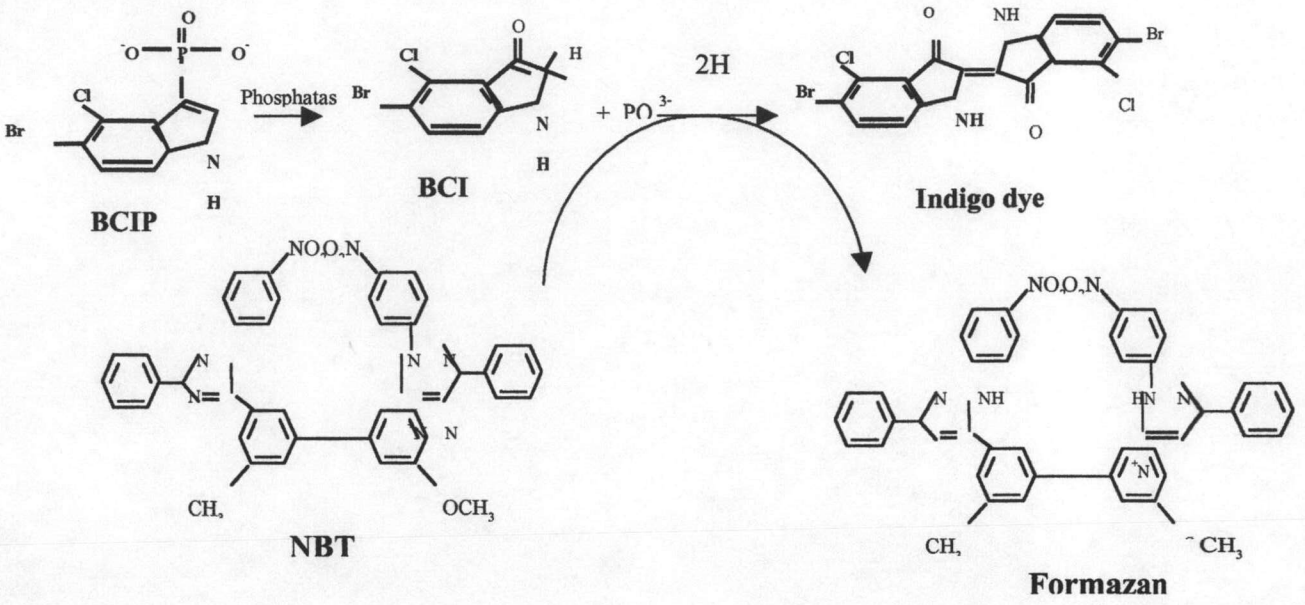


Figure 7. The phosphatase hydrolysis of BCIP is coupled to the reduction of NBT, yielding a formazan and indigo dye those are in black-purple.

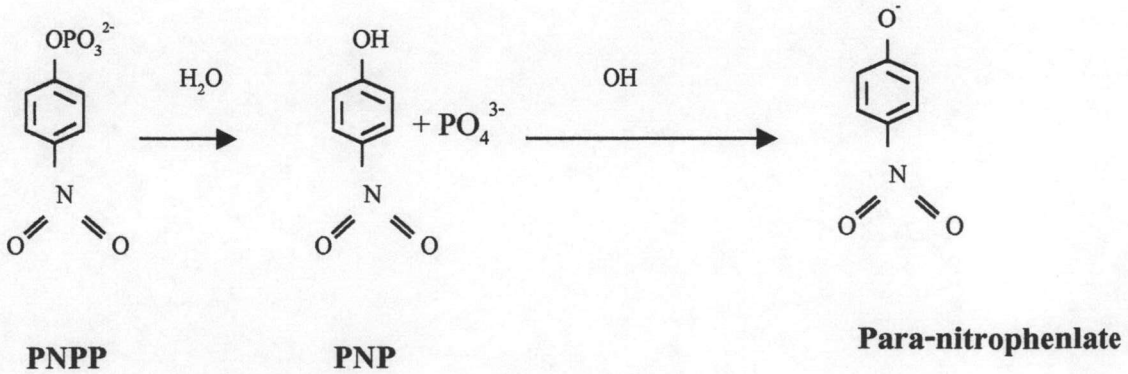


Figure 8. The phosphatase hydrolysis converts PNPP to PNP which provides the yellow color in basic condition.

AP in *A. mellifera*

In *A. mellifera*, little is known about AP. Most of researches in phosphatase trends to work on AC. The histochemical and cytochemical localization of AC has been used in an attempt to map the sites of cellular lysis and death. Free acid and AP activities are found in the basal area of the midgut epithelial cells and the former also occurred in the haemocoel (Gregorc and Bowen, 1997).

The enzyme activity seems to become enhanced in developing and differentiating columnar cells as the basal plasma membrane. Some biochemical experiments have also been made on the absorptive capacity of the midgut epithelium of adult honeybee, *A. mellifera*, in relation to carbohydrates (Crailsheim, 1988a) and amino acid (Crailsheim, 1988b).

A type of secretion is also significant in the epithelial cells of the adult bee midgut (Jimenez and Gilliam, 1990). AC activity is additionally located in somatic and germ cells of differentiating honeybee worker ovaries and in midgut cells of metamorphosis bees. It shows the intensity and distribution of electron dense deposits of lead phosphate during different phases of post-embryonic bee development (Maraes and Cavalcante, 2002). AC can be used as a marker of lytic activity in the cell. Free in the cytoplasm, it is an indicator of cellular autolysis. In different insects including *A. mellifera*, the activity of AC and AP has been histochemically visualized (Jimenez and Gilliam, 1990; Jones and Bowen, 1993).

AC and AP activity is found in different tissues in developing honeybee larvae. Results of light microscopy, acid phosphatase activity is found in the midgut of honeybee larvae of all ages. Characteristically, the enzymatic activity is found in the area of the brush border and in the basal area of epithelial cells. In the posterior part of the midgut, the activity is also found in secretory cells. This activity is involved in the development of the peritrophic membrane. In these cells, the apical membrane bound activity was insignificant (Gregorc and Bowen, 1997).

AP in other organisms

In many species of insects, the enzyme is found in midgut, hindgut, malpighian tubule, brain, and free enzyme in cytosol of cell. Most of researches in AP from several organisms is involved in the property of enzyme, cytochemistry, histochemistry, and gene expression (Yang *et al.*, 2000)

- AP in *E. coli*

In *E. coli*, AP in the periplasmic space is active. This is a metalloenzyme containing such as Zn^{2+}, Mg^{2+}, etc. AP is a dimeric enzyme (Halfold *et al.*, 1969). Characterization of Mg^{2+} content and the function of these ions will likely provide important and missing links in defining the mechanism of action of AP in *E. coli* (Anderson *et al.*, 1975). The *PhoA* gene of *E. coli* encodes AP that occurs in three forms of isoenzymes. Each isoenzyme depends on the growth condition of cells. In addition, each isozyme of AP was purified and characterized (Bradshaw *et al.*, 1981).

- AP in human

The localization of AP is in the wall of the blood vessels and at the synaptic regions. AP may be related to the alteration of permeability and supply of free energy. Hydrolytic AP activity increases in senescent cells. Histochemistry of AP in brain and spinal cord in human shows the pattern of reaction either in the form of fine beads or larger knobs around the cells and along their processes. Some neurons show patches of intensely positive reaction in their cytoplasm which may indicate the presence of the enzyme in lipofuscin pigments (Nandy and Bourne, 1963). Membrane-bound glycoproteins appear to be the products of at least three related gene loci. Four different AP isozymes are found in mammals. The placental, placental like germ cell, and intestinal isoenzymes are tissue specific. In contrast, tissue non-specific isozyme is found in liver, bone, or kidney (Henthorn *et al.*, 1987). The cDNA library of AP in liver/bone/kidney contains 2,533 bp. An open reading frame encodes 524 amino acid polypeptide with predicted molecular mass of 57.2 kDa. The deduced liver/bone/kidney *AP* precursor polypeptide shows 52% homology to human placental

AP and 25% homology to *E. coli AP* (Weiss *et al.*, 1986). The cDNA library clone for adult human intestinal *AP* was isolated from λgt11. The clone insert of 2,513 bp can encode 528 amino acid. The intestinal AP amino acid shows 86.5% identity to placental and 56.6% identity to liver/bone/kidney *AP* (Henthorn *et al.*, 1987). Three closely related AP genes reside on chromosome 2 in human. One of these encodes the classic heat-stable placental *AP* (*ALP1*). The other is related to *ALP1* encoding placental AP like enzyme in the testis and thymus. The third gene encodes intestinal *AP*. *ALP1* and intestinal *AP* are highly tissue specific in spilt of nearly 90% of sequence similarity (Brain *et al.*, 1988).

- AP in *M. domestica*

The activities of AP in house files are determined at different ages. Analyses are based on the hydrolysis of beta-napthol phosphate. AP activity is the highest in 2 day-old larvae. The AP activity builds to a high level in young larvae but it decreases to a low level by the time of puparium formation. It remains low and is almost constant throughout the adult stage (Barker and Alexander, 1958).

- AP in *P. americana*

The activity of AP is encountered in the mushroom bodies, central body, antennal glomeruli, and specific part of some neural connections including the optic nerve, antennal nerve, circumoesophageal connectives, and nerves leaving the suboesophageal ganglion. Native polyacrylamide gradient electrophoresis suggests that AP activity is present as multiple isozymic forms which show up in the 120-130 kDa range of standard protein (Verhaert *et al.*, 1990).

- AP in *S. americana*

The expression of epithelial AP in segmental grasshopper is associated with segmentation of limb in different developmental stages. The proper pH range of epithelial AP is 9.5 in diethanolamine buffer. The grasshopper epithelial AP is a single protein or a small group of isoenzymes which electrophoretic mobility is like of cockroach. Epithelial AP shows strong activity at epithelial cells

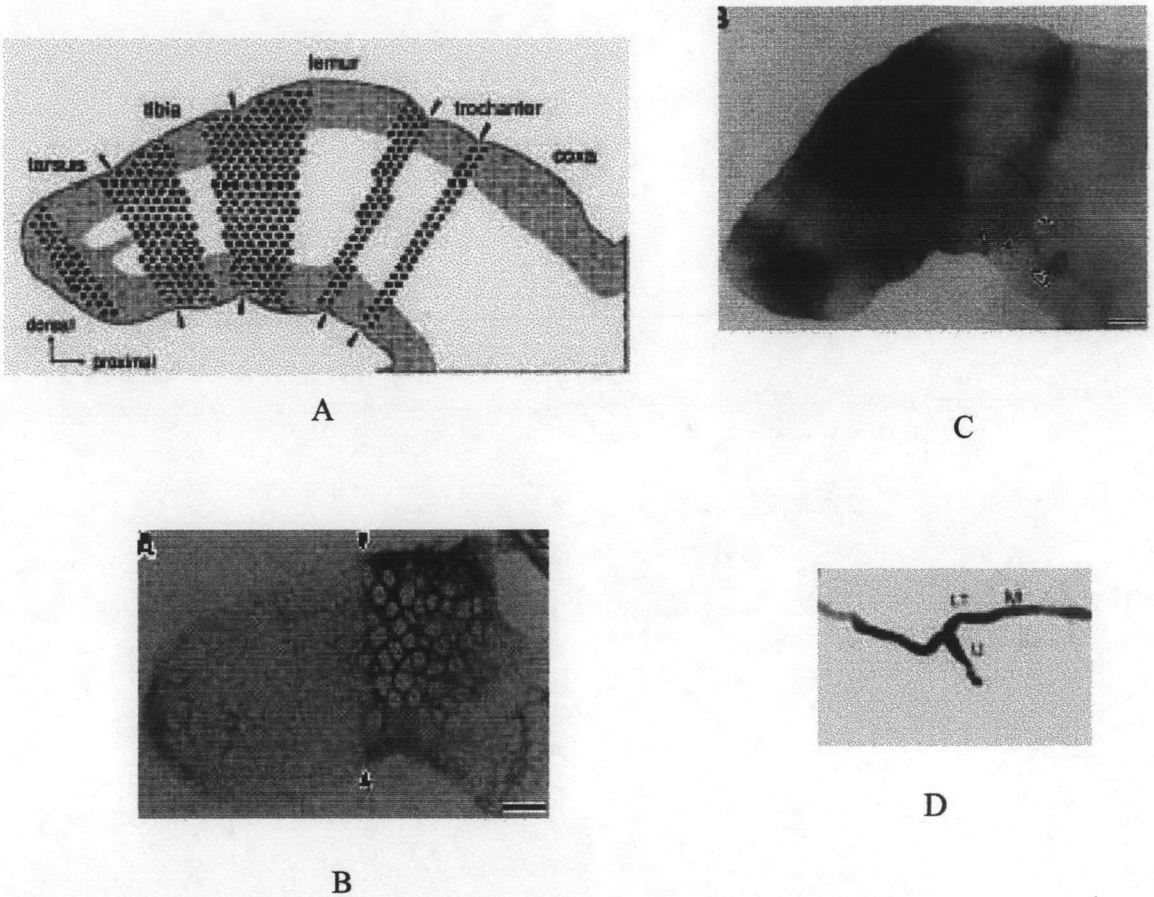
invaginating and extending during the formation of apodemes. The histochemistry also shows the AP activity in other appendages, in antennae similar to the limb, weakly in mouthparts, and in terminal cerci. The first detection of AP activity is in limb buds. After the appendages elongate and become segmented, the positive activity is found in head. Due to the examination of AP from all developmental stages, the activity band is completely extended in epithelial cells around a limb. The pattern of expression of epithelial AP is consistent with a function in epithelial cells or extracellular matrix that occurs during cell movements and rearrangements in limb morphogenesis. All of these are regions of epithelial shape changes including limb bud evagination, segment boundary invagination, and apodeme extension into the limb interior (Figure 9A, 9B, and 9C) (Chang *et al.*, 1993).

- AP in *D. melanogaster*

The discovery of gene control of this enzyme in *D. melanogaster* larvae raises the possibility of using this system for the studies of enzyme regulation in a diploid organism. Electrophoretic study of AP in developing *Drosophila* shows that different stages are characterized by the appearance and disappearance of organ-specific enzyme bands. In histochemistry study of developing *Drosophila*, AP was first seen in eggs after fertilization and is localized to hypodermis at that time in instar larvae. AP in *Drosophila* seems to be a family of enzymes whose members are organ or even tissue specific (Schneiderman *et al.* 1966).

AP activity is found in ellipsoid body ring neuron in the adult brain, malpighian tubule, ureter, and other lower tubule of *D. melanogater* (Figure 9D). Studying in gene expression of AP indicates four isoforms of AP, *Aph-1*, *Aph-2*, *Aph-3*, and recently found *Aph-4*. The genomic DNA of *Aph-4* in *D. melanogaster* is also completely sequenced. The length of 1,952 bp of cDNA contains a long open reading frame of 578 amino acid. That contains the highly conserved amino acid of AP. The level of *Aph-4* mRNA is the highest in larval and adult stages. From the sequence data, it shows that *Aph-4* is the most closely related to tissue

non specific *AP* in vertebrates. The only other insect *AP* sequence available from *B. mori* is 47% identical in similarity (Figure 10) (Yang *et al.*, 2000).



A

C

B

D

Figure 9. A schematic diagram depicting the five circumferential bands or epithelial AP in epithelial cells of grasshopper limb (A). Cellular distribution of AP activity in sectioned metathoracic limbs of grasshopper (B). AP activity that converts NBT/BCIP substrate and gives blue color in limbs (C) (Change *et al.*, 1993). Activity of AP in malpighian tubule and lower tubule of *D. melanogaster* (D) (Yang *et al.*, 2000).

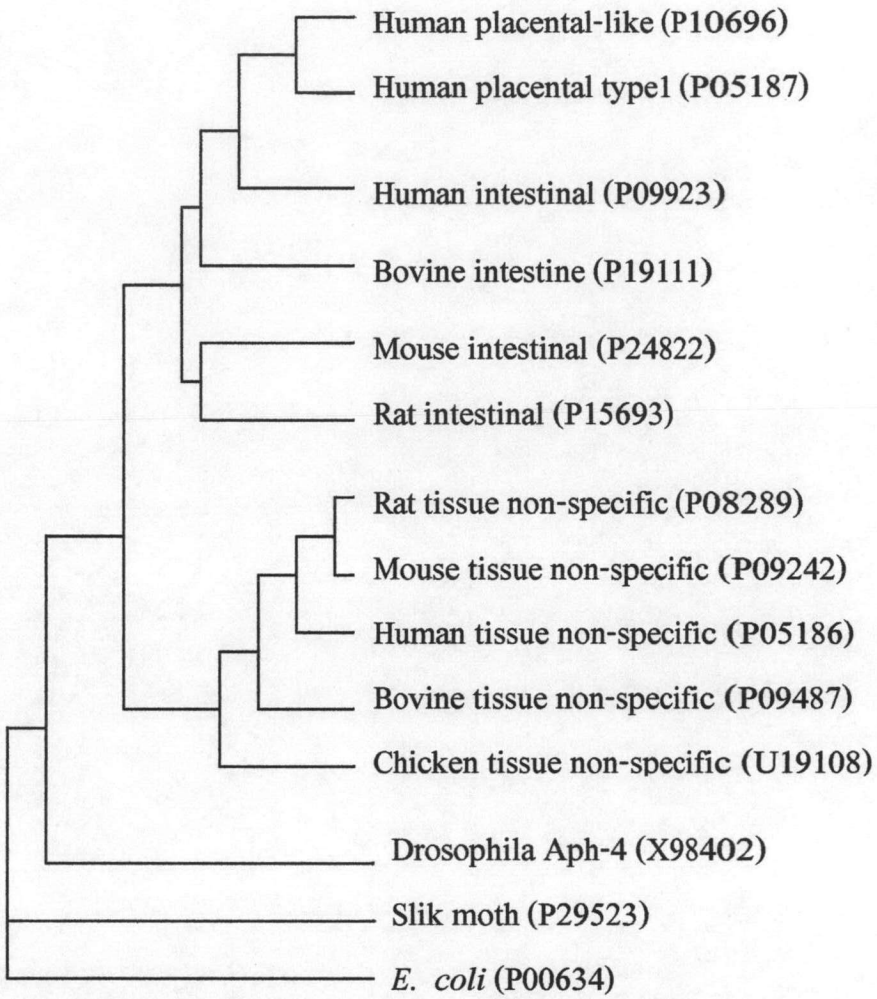


Figure 10. Phylogenic tree of *AP* in many organisms; bacteria, insect, chick, and mammal (Yang *et al.*, 2000).