OSAKA UNIVERSITY

OSAKA

# STUDIES ON DEVELOPMENTAL AND EVOLUTIONAL ASPECT OF INSECT CYTOCHROMES C

A Dissertation for a Doctorate of Science

by

SEIJI INOUE

ACKNOWLEGMENT

I wish to thank Prof. T. Yamanaka (Department of Chemistry, Faculty of Science, Tokyo Institute of Technology) for suggesting this work as well as for constant guidance in the course of the work, and Prof. H. Matsubara for his interest and encouragement during this work. I also thank Dr. T. Hiroyoshi, Dr. H. Inoue and Miss R. Wada (Department of Genetics, Medical School, Osaka University) for cultivation of houseflies and fruit flies, Dr. H. Ohgami (Central Institute, Japan Insecticide MGF. Co.) for cultivation of flesh flies and Mr. M. Matsui (Wakayama Pref.) for his generosity in supplying honeybees. I also thank Dr. S. Wakabayashi and Mr. Y. Yao for their valuable technical advices for sequence studies, Dr. Y. Fukumori (Department of Chemistry, Faculty of Science, Tokyo Institute of Technology) for technical advices for preparation of cytochrome c oxidase, Dr. K. Wada, Dr. T. Hase and Mr. Y. Takahashi for their suggestions and discussions and many laboratory colleagues for their encouragements.

# ABBREVIATIONS

| | |
|---|---|
| Cm- | S-Carboxymethyl- |
| Cmc | S-Carboxymethyl cysteine |
| CPase | Carboxypeptidase |
| C-terminal | Carboxyl terminal |
| EDTA | Ethylenediaminetetraacetic acid |
| HPLC | High-performance liquid chromatography |
| N-terminal | Amino terminal |
| PMSF | Phenylmethylsulfonylfluoride |
| PTH | Phenylthiohydantoin |
| SDS | Sodium dodecylsulphate |
| TLC | Thin layer chromatography |
| TPCK-trypsin | L-1-Tosylamido-2-phenylethyl chromethyl ketone-treated trypsin |

CONTENT

# I. INTRODUCTION

Cytochrome c is one of the proteins which have extensively been investigated in terms of its chemistry, structure, function, genetics and evolution. It acts as the electron carrier between complex III and complex IV of the mitochondrial electron transport chain. It occurs in all eukaryotes, more than 80 eukaryotic cytochromes c have been sequenced [1-4], and their evolutionary relationships have extensively been studied [5-9]. In spite of such extensive studies, isocytochromes c, which are "isoenzymes" of cytochrome c, were found only in yeast [10, 11] and mouse [12]. We found that isocytochromes c (larval-type and adult-type cytochromes c) exist also in the housefly Musca domestica L. [13]. Multiple forms of cytochrome c which have already been reported are summarized in Table I. The amounts of Larval- and adult-type cytochromes c varied during metamorphosis of the housefly [14]. Developmental variation and tissue distribution of two isocytochromes c of the housefly may give an important information to understand the mechanism of metamorphosis. The amino acid sequence studies may also give an important information about the evolutional process of insects. In this study, larval- and adult-type cytochromes c of the housefly [15] and cytochromes c of the fruit fly, the flesh fly and the honeybee [16] were sequenced and their evolutionary relationships were discussed.

Isocytochromes c

| Organisms | Type | Amino acid difference | Remarks | References |
|---|---|---|---|---|
| Yeast | iso-1-cytochrome c<br>iso-2-cytochrome c | 21 | Varied with growth condition | Sherman et al. [10]<br>Sels et al. [11] |
| Mouse | testis-specific and somatic cytochromes c | 13 | Tissue specific | Hennig [12] |
| Rat | | n.d. | | Kim [75] |
| Rabbit | | n.d. | | Inoue et al. (unpublished) |
| Housefly | larval- and adult-type cytochromes c | 6 | Varied during metamorphosis | Yamanaka et al. [13]<br>Inoue et al. [15] |

Allelic variations

| Organisms | Amino acid difference | Occurrence | References |
|---|---|---|---|
| Human | 1 | 2 % | Matsubara et al. [76] |
| Hippopotamus | 1 | 13 % | Thompson et al. [77] |
| Carp | 1 | ca. 50 % | Gürtler et al. [78] |

Heterozygous variations

| Organisms | Amino acid difference | Remarks | References |
|---|---|---|---|
| Hinny | 1 | Horse cyt c : Donkey cyt c = 1 : 1 | Walasek et al. [79] |
| Mule | 1 | | |

Modifications

| Organisms | Amino acid difference | Remarks | References |
|---|---|---|---|
| Neurospora | 1 | Trimethylation | Scott et al. [80] |
| Rat | 1-3 | Deamidation | Flatmark et al. [81] |
| Beef | | | Flatmark [82] |

Table I. Summary of multiple forms of cytochrome c already reported.

# II. BIOLOGY AND BIOCHEMISTRY OF INSECT CYTOCHROMES c

# 1. ISOCYTOCHROMES c OF THE HOUSEFLY

## (a) OCCURRENCE OF TWO ISOCYTOCHROMES c

### 1) MATERIALS AND METHODS

#### Materials

The housefly Musca domestica L. were reared at 25°C. Third inster larvae, pupae at the early stage and adults were stored at -20°C until use. Chemicals used were reagent grade and purchased from Wako Pure Chemical Industries, Ltd. (Osaka) and Nakarai Chemicals, Ltd. (Kyoto).

#### Purification of Cytochrome c

Cytochrome c was separately purified from 3rd inster larvae, pupae and adults of the housefly according to Yamanaka et al. [17] with some modifications. Insects were homogenized with sand in 10 mM EDTA containing 10 μM PMSF. Homogenate obtained was acidified to pH 4.5 with acetic acid and allowed to stand for 2 h. Then it was neutralized with ammonia and centrifuged at 10,000 x g for 20 min. The resulting supernatant was dialyzed against 10 mM EDTA containg 10 μM PMSF, filtrated with celite, subjected to chromatography with an Amberlite CG-50 column equilibrated with 20 mM ammonium phosphate buffer (pH 6.8) and cytochrome c adsorbed on the column was eluted with 500 mM ammonium phosphate buffer (pH 6.8). The red-coloured eluate was

90% saturated with ammonium sulphate and centrifuged at 20,000 x g for 20 min. The resulting supernatant was charged on a DEAE-cellulose column which had been equilibrated with 90% ammonium sulphate solution. The cytochrome c adsorbed on the column was eluted with water and then eluate was dialyzed against 20 mM ammonium phosphate buffer (pH 6.8). The dialysate was charged on an Amberlite CG-50 column (20 x 150 mm) and cytochrome c adsorbed on the column was eluted with a linear gradient from 20 mM to 300 mM ammonium phosphate buffer.

Polyacrylamide Gel Electrophoresis

Disc electrophoresis at a running pH of 6.6 was performed on 15% polyacrylamide gel according to Flatmark [18] with some modifications. Separation gel was prepared from 2 volumes of glycine solution (20% glycine and 0.75% (v/v) N,N,N',N'-tetramethyl-ethylenediamine, pH 8.3), 1 volume of acrylamide solution (60% acrylamide and 2.5% N,N'-methylene-bisacrylamide) and 1 volume of 0.02% ammonium persulphate. Stacking gel was made by adding a suitable amount of dry Sephadex G-200 powder to the solution which was prepared from 1 volume of $NH_4OH$-glycine buffer (0.48 N $NH_4OH$ and 4.8% glycine, pH 9.6) and 2 volumes of 20 mM ammonium phosphate buffer (pH 6.9). To prepare the sample gel, cytochrome c solution which was prepared from 1 volume of $NH_4OH$-glycine buffer and 2 volumes of cytochrome c in 20 mM ammonium phosphate buffer was charged on the stacking gel, and then dry Sephadex G-200 was added to the cytochrome solution. The tray buffer (pH 8.3) was composed of 1.37% glycine and 3.82% (v/v) 2.6-lutidine. The electrophoresis was run at room

temparature and a constant current of 2.5 mA/tube. After a 2-h electrophoresis, cytochrome c in the gel was monitored by absorbance at 415 nm using a Gilford spectrophotometer model 250 equipped with a gel scanner apparatus. The positions of the bands where cytochrome c was located in the gel were also checked by protein staining and heme staining [19].

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn [20] with slight modifications. Slabbed gels were used as described by Wada and Snell [21]. The gel slab, 14.3 x 10 x 0.1 cm, was made by polymerization of 10% acrylamide and soaked overnight in 100 mM Tris-acetate buffer, pH 8.2, containing 0.1% SDS. Samples were dissolved in 25 mM Tris-acetate buffer containing 1% SDS and 1% $\beta$-mercaptoethanol and heated at 100$^{o}$C for 1 min to make the SDS-protein complex. Electrophoresis was carried out at 25 mA and 150 volt for 2-3 h at 20$^{o}$C. Proteins in the gel were stained by Coomassie brilliant blue R-250.

Amino Acid Analysis

The amino acid compositions of cytochromes c were determined with an amino acid analyzer (IRICA Instruments, Inc., Model A-3300) essentially according to the method of Spackman et al. [22]. Proteins were hydrolyzed in evacuated sealed tube for 24 h and 72 h at 110$^{o}$C with 5.7 N HCl.

## 2) RESULTS AND DISCUSSION

The profiles of chromatography of cytochrome c with Amberlite CG-50 column were shown in Fig. II-1. It seemed that two isocytochromes c were present in various stages of the housefly. Major cytochrome c in larvae and pupae was named as larval-type cytochrome c and that in adults as adult-type cytochrome c. Both kinds of cytochromes c were spectrophotometrically pure, as judged by the ratio of the absorbance at 550 nm in the reduced form to the absorbance at 280 nm in the oxidized form. No difference was obtained between larval-type and adult-type cytochromes c with respect to the absorption spectrum.

As shown in Fig. II-2, when an equimolar mixture of the two isocytochromes c which had been purified separately was subjected to polyacrylamide gel electrophoresis at pH 6.6, they were separated from each other. The adult protein migrated to the cathode more rapidly than the larval protein. This agrees with the results of an Amberlite CG-50 column chromatography and indicates that adult protein is more basic than larval one. Gel electrophoresis in the presence of SDS showed that both proteins had the same mobilities which corresponded to 12,000 daltons (data not shown). From the results mentioned above, it was deduced that larval-type and adult-type cytochromes c were same in sized but not in total charge.

The amino acid compositions of the two isocytochromes showed more clearly that they were different from each other (Table II-I). The adult-type protein contained one more residue each of glutamic acid and isoleucine than larval-type protein, while the latter had one more residue each of alanine and leucine than the

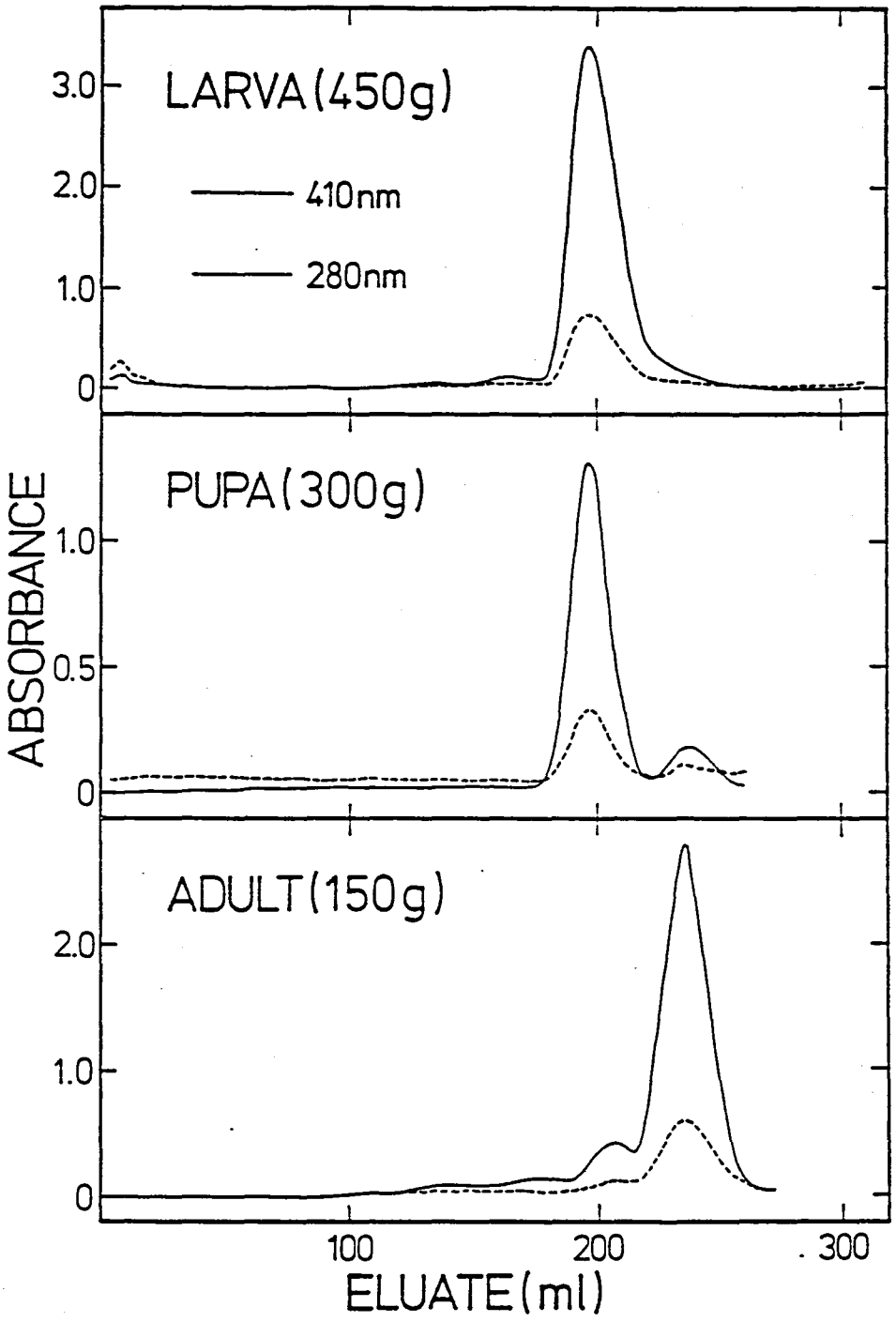


Fig. II-1. Elution patterns of cytochromes c at various metamorphic stages during the chromatography on an Amberlite CG-50 column. Cytochromes c were eluted with a linear gradient from 20 mM to 300 mM ammonium phosphate buffer.

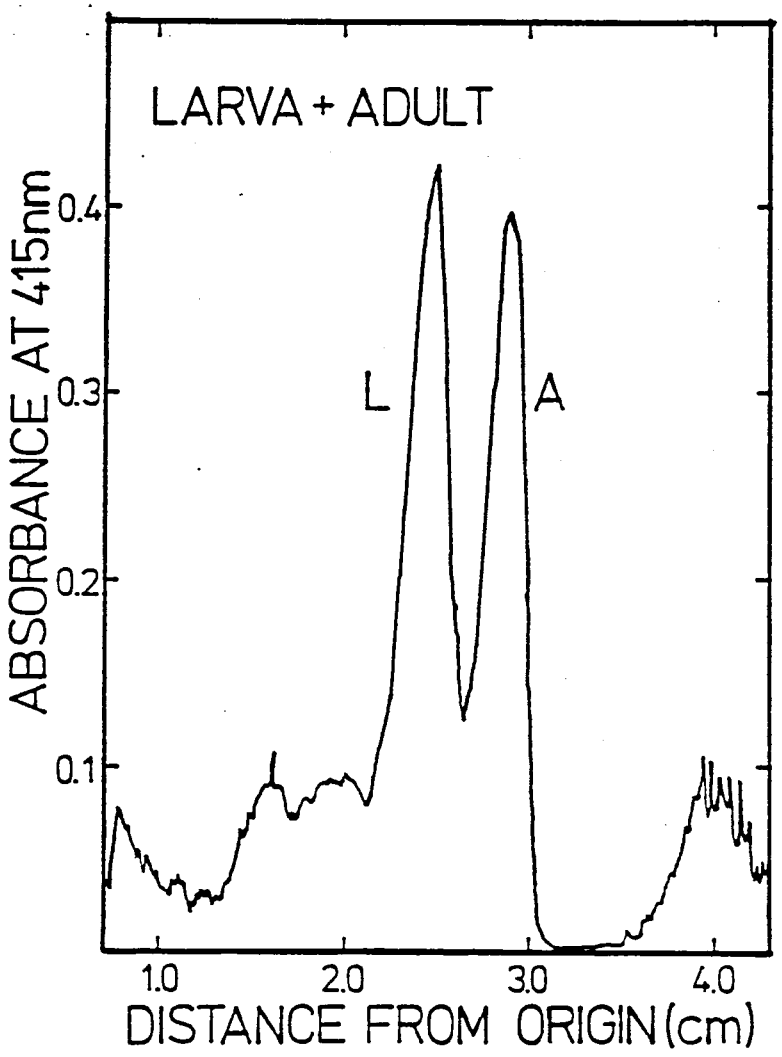


Fig. II-2. Separation of two isocytochromes c by polyacrylamide gel electrophoresis. Larval- and adult-type cytochromes c were purified separately from 3rd inster larvae and adults of the housefly, respectively, to an electrophoretically homogeneous state, and an equimolar mixture of two isocytochromes c was subjected to a disc electrophoresis. The cytochromes in the gel were monitored by the absorbance at 415 nm using a Gilford spectrophotometer model 250 equipped with a gel scanner apparatus. L, larval-type cytochrome c; A, adult-type cytochrome c.

| Amino acid | LARVA | | ADULT | |
|---|---|---|---|---|
| | Analysis | Sequence* | Analysis | Sequence* |
| Asp | 9.48 | 9 | 9.48 | 9 |
| Thr | 7.04 | 7 | 6.90 | 7 |
| Ser | 2.30 | 2 | 1.57 | 1 |
| Glu | 8.94 | 8 | 10.0 | 9 |
| Pro | 5.06 | 5 | 4.88 | 5 |
| Gly | 14.2 | 14 | 14.1 | 14 |
| Ala | 12.2 | 12 | 11.1 | 11 |
| Cys | | 2 | | 2 |
| Val | 5.01 | 5 | 5.15 | 5 |
| Met | 0.81 | 1 | 0.79 | 1 |
| Ile | 3.89 | 4 | 4.89 | 5 |
| Leu | 7.59 | 8 | 6.87 | 7 |
| Tyr | 3.42 | 4 | 3.43 | 4 |
| Phe | 4.73 | 5 | 4.66 | 5 |
| Lys | 14.0 | 14 | 14.4 | 15 |
| His | 3.09 | 3 | 3.19 | 3 |
| Arg | 2.67 | 3 | 2.88 | 3 |
| Trp | | 1 | | 1 |
| Total | | 107 | | 107 |

Table II-I. Amino acid compositions of larval- and adult-type cytochromes c of housefly. Values are calculated from averages of duplicate analyses of one 24-h and one 72-h hydrolysate. * Based on amino acid sequences described in section III-1.

former. The compositions were in satisfactory agreement with those obtained from the sequence as described in section III.1 and the structural difference between larval- and adult-type cytochromes c will be further described there.

## (b) DEVELOPMENTAL VARIATION OF ISOCYTOCHROMES C OF THE HOUSEFLY

### 1) MATERIALS AND METHODS

#### Materials

The housefly Musca domestica L. were reared at 25°C with heat-treated yeast cells as a feed.

#### Quantification of Two Isocytochromes c During the Metamorphosis of the Housefly

Cytochrome c was partially purified from the housefly (approx. 30 insects) at various stages by the method of Williams et al. [23]. Insects were homogenized in 0.5% $Al_2(SO_4)_3$, pH 4.5, and each homegenate was allowed to stand for 2 h at room temperature. The homogenate was centrifuged at 20,000 x g for 15 min, the resulting supernatant was neutralized with 1 M $NH_4OH$ and re-centrifuged to remove the precipitate appeared. The supernatant thus obtained was charged on a small Amberlite CG-50 column and cytochrome c was eluted with 20 mM sodium phosphate buffer, pH 8.0, containing 500 mM NaCl. The volume of this

eluate was adjusted to 1.0 ml and the amount of cytochrome c recovered was calculated by measuring the absorbance at 550 nm of its fully reduced form using a millimolar extiction coefficient of 27.7 [24]. The preparation obtained was desalted by the gel filtration with a Sephadex G-25 column which had been equilibrated with 100 mM $NH_4HCO_3$, lyophilized and subjected to gel electrophoresis at a running pH of 6.6 as reported already [14]. Cytochrome c in the gel was monitored by absorbance at 415 nm using a Gilford spectrophotometer model 250 equipped with a gel scanner apparatus.

## 2) RESULTS

The amounts of the two isocytochromes c were separately determined by electrophoresis as previously described [14]. A quantitative variation in the isocytochrome c content during the metamorphosis is shown in Fig. II-3. In larvae, the amount of larval-type cytochrome c was approximately 90% of the total amount of cytochrome c and the remaining 10% was the adult-type cytochrome, while in pupae at the early stage the amount of the larval-type cytochrome decreased approximately by 50% but that of the adult-type cytochrome remained the same as that in larvae. At the late stage of pupa the amount of adult-type cytochrome c increased 3.7-fold, while the amount of the larval-type cytochrome was approximately 50% of that at the early pupal stage. On adult emergence, the amount of adult-type cytochrome c further increased 6.3-fold as compared with that at the late

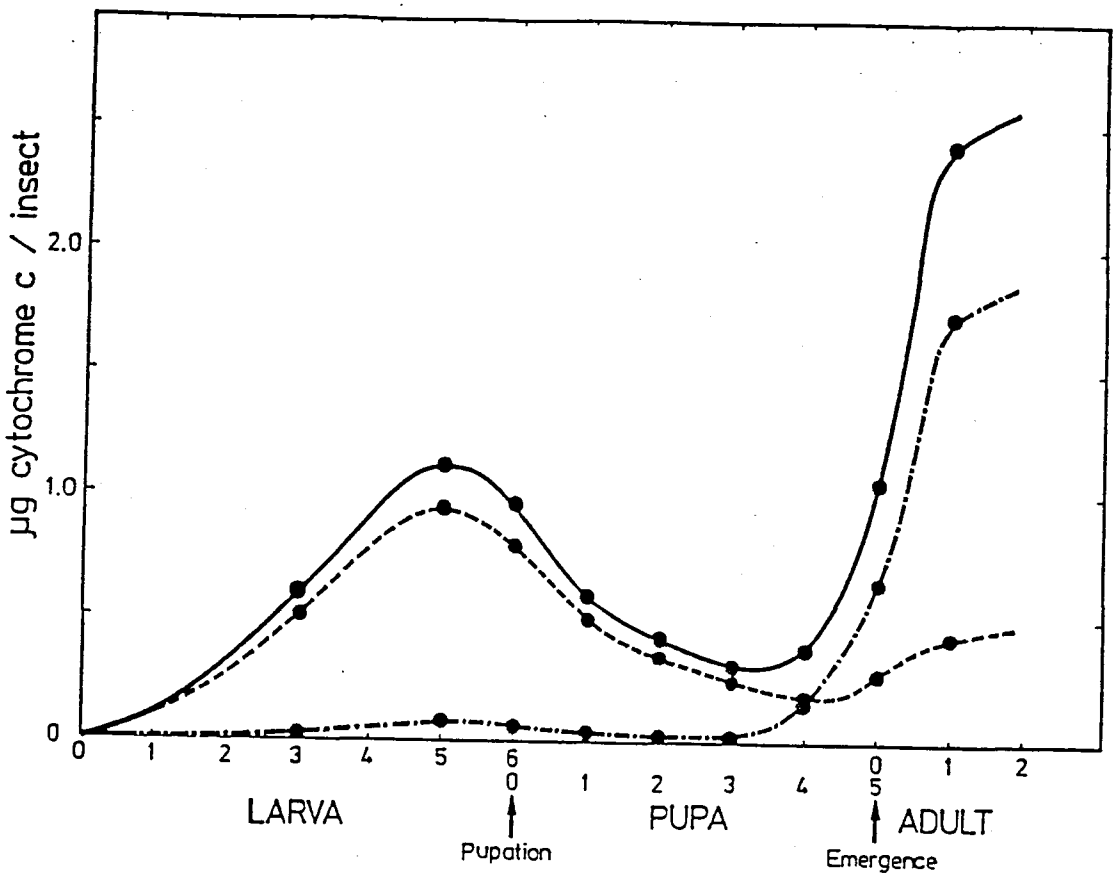


Fig. II-3. Variation in the cytochrome c content during the metamorphosis of the housefly. The amount of each isocytochrome c was estimated from the densitometric tracing by absorbance at 415 nm, and the total amount of cytochrome c was determined by the absorbance at 550 nm ($\varepsilon_{mM}$ at 550 nm = 27.7) after it was reduced by dithionite. Thirty to forty insects were used in each experiment. ———, total; ------, larval-type cytochrome c; ·—·—, adult-type cytochrome c.

pupal stage, while the amount of the larval-type cytochrome increased 2-fold. Consequently, the total amount of cytochrome c in adults was more than twice as much as that in pupae at the early stage. The increase in the total amount of cytochrome c at adult emergence was caused mainly by the increased biosynthesis of adult-type cytochrome c. Larval-type cytochrome c was found in the all developmental stages of the housefly, while the adult-type protein was not found in eggs and its content was very low in the larval stages.

## 3) DISCUSSION

The reason why two isocytochromes c occur in the housefly may be explicable in two ways as judged from the change in the amount of two isocytochromes c as shown in Fig. II-3.

One explanation is that larval-type cytochrome c may be specific for larval tissues while adult-type cytochrome c may be specific for adult tissues which are derived from imaginal disks. Tissue specific distribution of isocytochromes c was already reported in mouse [12]. Testis-specific cytochrome c was occurred only in testis but not in other tissues. The increase of adult-type cytochrome c during metamorphosis seems to be relative to the development of the imaginal disks; adult-type cytochrome c in larvae may be derived from the cytochrome c of the imaginal disks. The decrease in larval-type cytochrome c during the pupal stage seems to be relative to the destruction of the larval tissues. To make clear whether this explanation is

correct or not, the investigation of cytochrome c in the imaginal disks will be necessary.

Another explanation is that larval-type cytochrome c is constitutive while adult-type cytochrome c is adaptive. The amount of larval-type cytochrome c seems to be constant through the developmental stages of the housefly, while that of adult-type cytochrome c increases dramatically at adult emergence probably in order to supply the energy demand for flight.

Study on the tissue distribution of two isocytochromes c (section II.1.c) may give an explanation about their variation during metamorphosis of the housefly.

## (c) TISSUE DISTRIBUTION

### 1)MATERIALS AND METHODS

A Detailed Study of Tissue Distribution of Two Isocytochromes c

Each insect was dissected and each tissue was separated. Fat body (three insects), Malpighian tubules (30 insects), ganglion (100 insects), wing disks (100 insects), leg disks (100 insects), salivary glands (50 insects) and larval carcass (three insects) were collected from third-instar larvae. Flight muscles (one insect), legs (ten insects), Malpighian tubules (40 insects), testis (100 insects) and ovaries (20 insects) were collected from adults.

Each tissue obtained was homogenized in 0.1 ml of 0.5% $Al_2(SO_4)_3$ at pH 4.5, and the resulting homogenate was kept for 2 h at room temperature and then centrifuged. The resulting supernatant was neutralized by 1 M $NH_4OH$ and then centrifuged to discard aluminum hydroxide appeared. The supernatant thus obtained was dialyzed against 20 mM ammonium phosphate buffer (pH 6.8), containing 40% sucrose, for 3 h. The samples thus concentrated were directly subjected to slab gel electrophoresis at a running pH of 6.6. About 1 g of methyl green was added to the upper tray buffer (500 ml) in order to prevent the adsorption of cytochrome c to polyacrylamide gel. Proteins in the gel were detected by silver staining [25].

Quantification of Two Isocytochromes c as a Function of Different Body Parts of Adult Houseflies

To determine the amounts of isocytochromes in different body parts, adult flies (100 insects) were treated with acetone which had been chilled at -20°C until water in the tissues was replaced by acetone and dried by evacuation. Each insect was separated into head, thorax and abdomen and cytochrome c was prepared from each group of the tissues, by the method of Williams et al. [23].

## 3) RESULTS

Distribution in tissues of the two isocytochromes was determined in detail as shown in Figs. II-4A and B. Cytochrome c from salivary glands of larvae, Malpighian tubules of larvae and

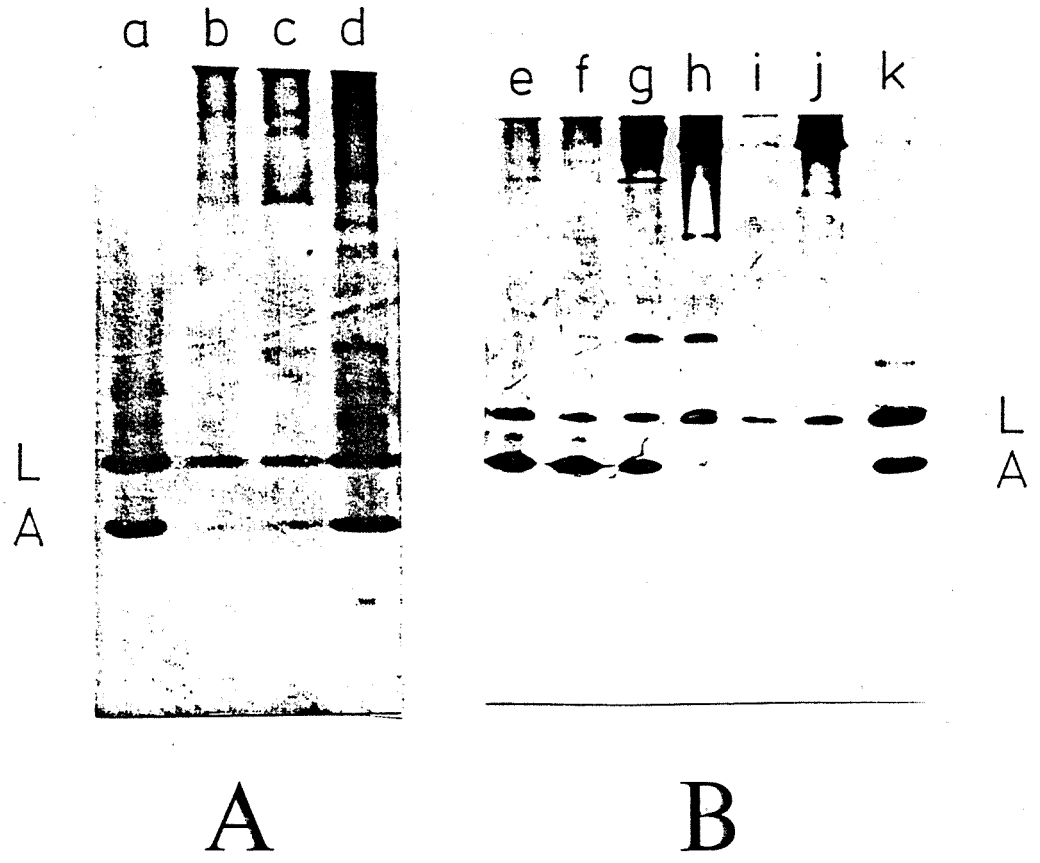


Fig. II-4. Electrophoretic patterns of cytochrome c from various tissues of larval (A) and adult (B) flies. Proteins were detected by silver staining. Lanes a and k, a mixture of equal amounts of purified larval- and adult-type cytochromes c; lane b, wing disks (100 insects); lane c, leg disks (100 insects); lane d, ganglion (100 insects); lane e, male flight muscles (one insect); lane f, female flight muscles (one insect); lane g, legs (ten insects); lane h, Malpighian tubules (40 insects); lane i, testes (100 insects); lane j, ovaries (20 insects). L, larval-type cytochrome c; A, adult-type cytochrome c.

larval carcass was mainly larval-type (data not shown). Imaginal disks contained mainly larval-type cytochrome c (lanes b and c in Fig. II-4A). Ganglion of larvae contained appoximate equiamount of larval-type and adult-type cytochromes c (lane d). Larval-type cytochrome c was major component (lanes h, i and j in Fig. II-4B) even in adult tissues, except in flight muscles and legs (lanes e, f and g), respiratory activity of which is very high.

As shown in Fig. II-5 and Table II-II, the amount of adult-type cytochrome c in thoraces was approximately 3.6 times as much as that of the larval-type cytochrome, while in abdomens the larval-type cytochrome was approximately three times as that of the adult-type cytochrome. The content of larval-type cytochrome c in head was approximately the same as that of the adult-type cytochrome. Further, it is clear from Table II-II that appoximately 90% of the total amount of cytochrome c occurred in thoraces of the adult houseflies and 85% of the protein in thoraces occurred in flight muscles.

## 3)DISCUSSION

The above results show that the flight muscle development has probably brought about the increase of adult-type cytochrome c at adult emergence and in turn the increase of the total amount of cytochrome c. During the flight muscle development, myoblasts originating from imaginal disks fuse with larval fibers to evolve into adult flight muscles. Present study indicates that cytochrome c from imaginal disks is mainly larval-type.

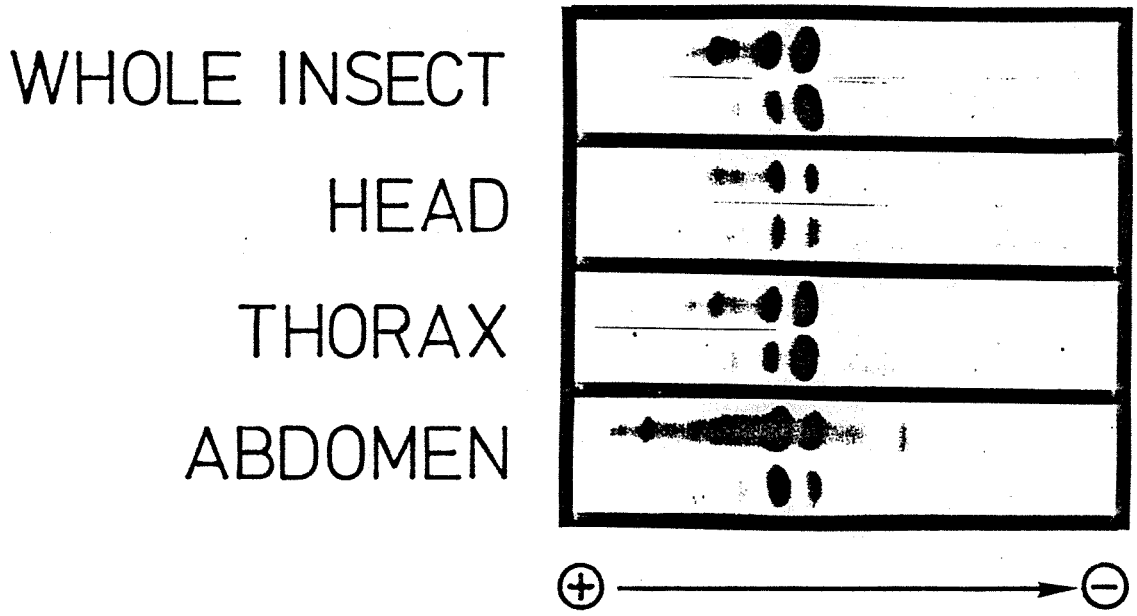


Fig. II-5. Electrophoretic patterns of cytochrome c from different body parts of adult houseflies. Gels were stained with protein staining (upper side in each block) or heme staining (lower side).

| Tissue | Content of cytochrome c (μg/insect) | | |
|---|---|---|---|
| | Larval type | Adult type | Total |
| Head | 0.049 | 0.044 | 0.111 |
| Thorax | 0.369 | 1.340 | 1.911 |
| Abdomen | 0.085 | 0.027 | 0.125 |
| Total | 0.503 | 1.411 | 2.147 |
| Whole insect | 0.477 | 1.498 | 2.171 |
| Flight muscle | 0.334 | 1.168 | 1.603 |

Table II-II. Distribution of two isocytochromes c as a function of different body parts of adult houseflies. The amount of each isocytochrome c was estimated from the areas under the cytochrome peaks on the densitometric tracings of the gels shown in Fig. II-5 and the total amount of cytochrome c was determined by the absorbance at 550 nm ($\varepsilon_{mM}$ at 550 nm = 27.7) after it was reduced by dithionite before being subjected to electrophoresis. Each value is an average of three different experiments. One hundred insects were used in each experiment.

Therefore, adult-type cytochrome c must be induced during the muscle development. Although cytochrome c is not obtained from fat body, it could be due to adsorption of the cytochrome to the precipitate during the preparation.

On the basis of developmental variation and tissue distribution of isocytochromes c, larval-type cytochrome c seems to be constitutive, while adult-type cytochrome c seems to be adaptive and genetically expressed to satisfy the energy demand such as flight.

## (d) REACTION WITH OXIDASE

### 1) MATERIALS AND METHODS

Materials

Horse heart cytochrome c was purchased from Sigma Chemical Co. (U.S.A.). Various chemicals were of reagent grade and purchased from Wako Pure Chemical Industries, Ltd. (Osaka) and Nakarai Chemicals, Ltd. (Kyoto).

Purification of Larval- and Adult-type Cytochromes c of the Housefly

To purify larval- and adult-type cytochromes c of the housefly, the insects were separately homogenized with 0.5% $Al_2(SO_4)_3$ [23]. After the homogenate was allowed to stand for 2

h, it was centrifuged at 20,000 x g for 20 min, pH of the resulting supernatant was adjusted to 8.0 with addition of 1 M $NH_4OH$, and then the solution was centrifuged at 20,000 x g for 20 min. The supernatant thus obtained was subjected to chromatography with an Amberlite CG-50 column equilibrated with 20 mM phosphate buffer (pH 8.0) and cytochrome c adsorbed on the column was eluted with 20 mM phosphate buffer (pH 8.0)/0.5 M NaCl. The eluate obtained was 90% saturated with ammonium sulphate and centrifuged at 20,000 x g for 20 min. The resulting supernatant was charged on a DEAE-cellulose column which had been equilibrated with 90% saturated ammonium sulphate solution. The cytochrome adsorbed on the column was eluted with 20 mM ammonium phosphate buffer (pH 6.8) and the resulting eluate which contained cytochrome c was dialyzed against the same buffer as used for the above elution. The dialyzed cytochrome c solution was charged on an Amberlite CG-50 column and cytochrome c adsorbed on the column was eluted with a linear gradient from 20 mM to 300 mM ammonium phosphate buffer (pH 6.8). The red-coloured eluate thus obtained was used as the purified cytochrome c preparation. About 4 mg of the larval-type cytochrome and about 10 mg of the adult-type cytochrome were obtained from 100 g each of larvae (third instar) and adult flies, respectively.

Preparation of Cytochrome c Oxidase

Cytochrome c oxidase was purified separately from larvae and adult of the housefly according to Jacob et al. [26]. About 60 g of larvae and 80 g of adults were homogenized in 50 mM Tris-HCl buffer, pH 7.4 containg 1 mM EDTA, 5 mM phenylthiourea and 0.25 M

sucrose, and in 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA and 0.25 M sucrose, respectively. The homegenate was centrifuged at 600 x g for 10 min. The supernatant thus obtained was centrifuged at 10,000 x g for 15 min. The mitochondrial pellet thus obtained was washed with the buffer used for the homogenization, suspended in the same buffer at a protein concentration of 25 mg / ml. To the resulting suspension was added 1 M potassium phosphate buffer (pH 7.4) to 0.2 M and further added 20% (w/v) Triton X-114 to the concentration of 1 mg Triton X-114 per mg of protein. The mixture thus obtained was centrifuged at 100,000 x g for 1 h. The resulting oily green pellet was washed with 0.2 M potassium phosphate buffer (pH 7.4) and suspended in 0.2 M potassium phosphate buffer (pH 7.4) containing 5% (w/v) Triton X-100. After the suspension obtained was diluted with 4 volumes of water, it was centrifuged at 100,000 x g for 1 h. The resulting supernatant was used as the partial preparation of cytochrome c oxidase to determine the reaction with cytochromes c. The supernatant obtained above was diluted by one volume of water, charged on DE-32 column, equilibrated with 20 mM potassium phosphate buffer (pH 7.4) containing 1% Triton X-100, and cytochrome c oxidase was eluted with 0.2 M potassium phosphate buffer containing 1% Triton X-100. To the eluate thus obtained was added saturated ammonium sulphate solution and 40% cholate to the final concentrations of 30% saturation and 3%, respectively. After being centrifuged at 20,000 x g for 30 min, the supernatant obtained was 40%-saturated by further addition of saturated ammonium sulphate solution , and then centrifuged at 20,000 x g for 30 min. The pellet obtained

was suspended in 0.1 M Tris-HCl (pH 8.0) containing 1% (w/v) Tween 20 and 0.2 M NaCl. The suspension was charged on a Sephadex G-200 column (1.5 x 20 cm) equilibrated with same buffer. Cytochrome c oxidase fraction of this eluate was used as the purified cytochrome c oxidase to be subjected to SDS polyacrylamide gel electrophoresis.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to Merle et al. [27]. The buffer system were the same as originally described by Laemmli [28]. Stacking gel contained 9.6% acrylamide, 0.3% N,N'-methylenebisacrylamide, 0.1 M Tris-HCl (pH 6.8), 0.1% sodium dodecylsulphate, 1 mg/ml ammonium sulphate and 1 μl/ml N,N,N',N'-tetramethylethylenediamine. The separation gel contained 16% acrylamide, 0.5% N,N'-methylenebisacrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% sodium dodecylsulphate, 1 mg/ml ammonium persulphate, 0.67 μl/ml N,N,N',N'-tetramethylethylene-diamine and 13% glycerol and 3.6 M urea. Stacking gel and separation gel were copolymerized as described by Cabral and Schatz [29]. To the samples were added equivolume of 5% sodium dodecyl sulphate, 5% β-mercaptoethanol, 20% glycerol and 20 mM sodium phosphate buffer (pH 6.5). Electrophoresis was carried out at constant voltage (75 V) for 16 h. Proteins in the gel were stained by Coomassie brilliant blue R-250.

Analytical Mesurement

Protein concentration was determined using Coomassie brilliant blue G-250 according to Bradford [30] with crystalline

bovine serum albumin as the standard.

Absorption spectra of cytochrome c oxidase were obtained with a Cary-16 recording spectrophotometer. A millimolar extinction coefficient $\Delta\varepsilon(A_{603nm}$ minus $A_{630nm}$; reduced) of 16.5 was used [31].

Activities of the larval and adult cytochrome c oxidase preparations were determined with a Hitachi spectrophotometer, model 220 A using a 1 cm light path cuvette; oxidation of ferrocytochrome c was followed by decrease in the absorbance at 550 nm with time. Cytochrome c oxidase activity is expressed as the first order velocity constant (K). Reactions were performed in 100 mM ammonium phosphate buffer, pH 7.4 or in 50 mM sodium phosphate buffer at various pH.

## 2) RESULTS

As shown in Fig. II-6, the spectral characteristics indicated that the purified preparations of cytochrome c oxidase were not significantly contaminated by any other cytochromes.

As shown in Fig. II-7, adult cytochrome c oxidase of the housefly showed the electrophoretic patterns similar to those of bovine cytochrome c oxidase on SDS polyacrylamide gel electrophoresis, except that some minor proteins were present as contaminants. Larval cytochrome c oxidase could not highly purified because its instability during purification. As purified cytochrome c oxidase had little activity, partially purified cytochrome c oxidase was used to determine the reaction

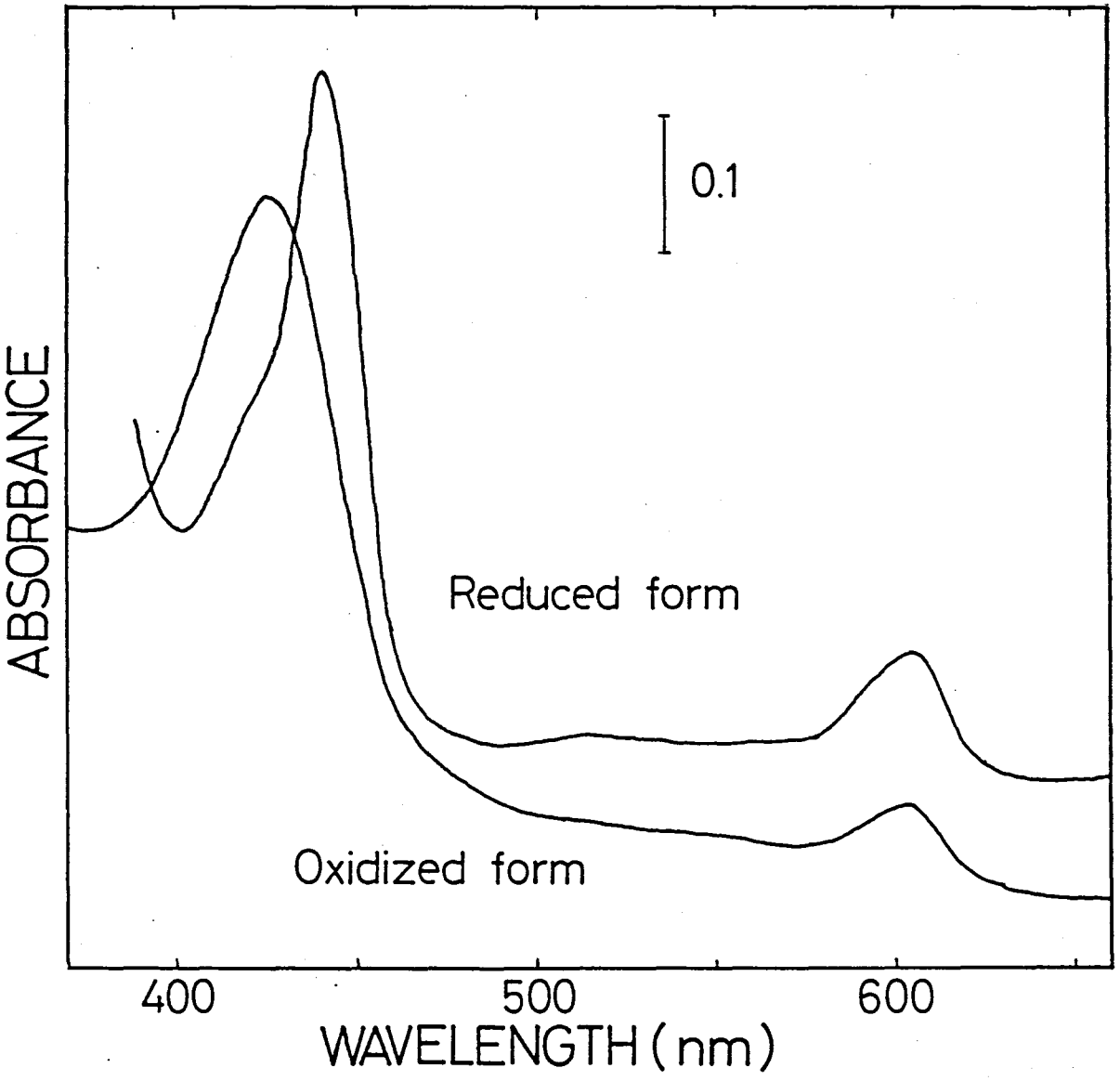


Fig. II-6. Absolute absorption spectra of the air oxidized and dithionite reduced cytochrome oxidase preparation of adult houseflies.

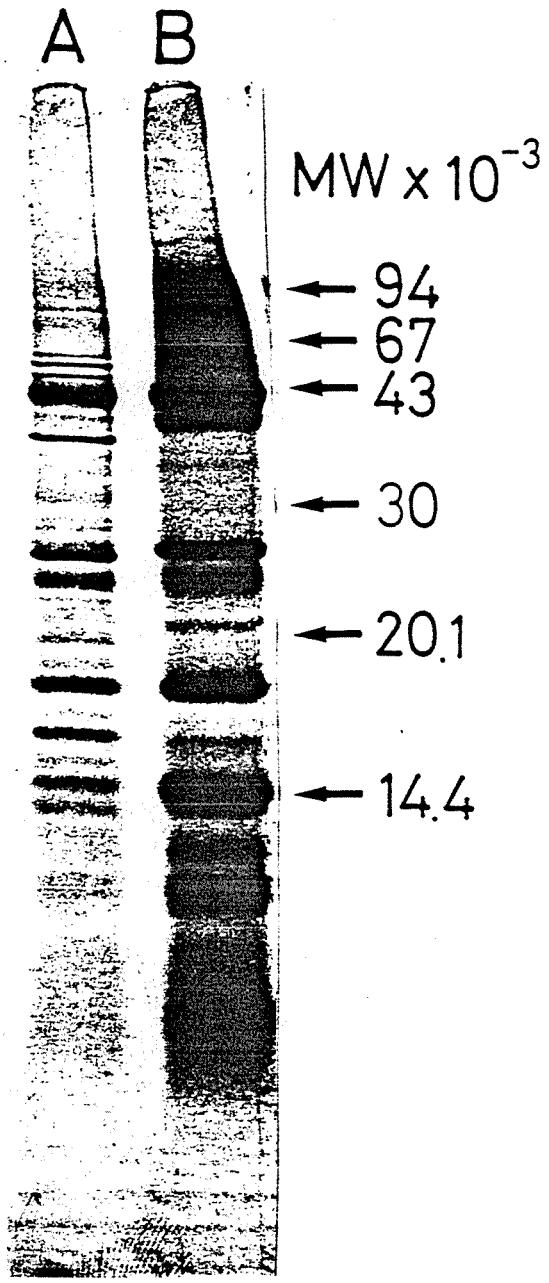


Fig. II-7. Comparison of the subunit composition of cytochrome c oxidases from adult housefly and from bovine heart. SDS gel electrophoresis was performed as described in "MATERIALS AND METHODS". Lane A, adult housefly enzyme; lane B, bovine heart enzyme.

with cytochrome c.

As shown in Table II-III, it was investigated whether any difference occurred between the larval and adult cytochrome c oxidase preparations in the reactivity with larval-type and adult-type cytochromes c. No significant difference was observed in their reactivity with the same cytochrome c. However, the reactivity with larval-type cytochrome c of each oxidase preparation was 1.5 times as high as that with adult-type cytochrome c.

The effect of pH on the cytochrome c oxidase activity of the adult enzyme is shown in Fig. II-8. The oxidase had the pH optimum of 6.2. A pH optimum for the larval oxidase was also around 6.2, but it could not be determined correctly, as the larval oxidase was very unstable.

## 3) DISCUSSION

As shown in Fig. II-7, adult cytochrome c oxidase had similar subunit composition to that of bovine cytochrome c oxidase. It has been already reported that cytochrome c oxidase of the locust is composed of seven subunits [32]. As larval cytochrome c oxidase was not purified to a homogeneous state, it remained unknown whether larval oxidase was identical with adult oxidase. It has been already reported that vertebrates contain tissue-specific isoenzymes of cytochrome c oxidase [33].

The reactivity of each oxidase preparation with larval-type cytochrome c was 1.5 times as high as that with adult-type

| Cytochrome c | Larval cytochrome oxidase | Adult cytochrome oxidase |
|---|---|---|
| ① Horse Heart | 100 (100) | 100 |
| ② Larval type | 133 (124) | 131 |
| ③ Adult type | 89 (87) | 90 |
| ②/③ | 1.49 (1.42) | 1.46 |

Table II-III. Relative reactivities with cytochromes c of larval and adult cytochrome c oxidase preparations. Reactions were measured in 100 mM ammonium phosphate buffer, pH 7.4 or 50 mM sodium phosphate buffer, pH 6.5 (values in parentheses). The reactivity is expressed as per cent of that of horse heart cytochrome c with each oxidase. Each value is an average of 4 experiments.

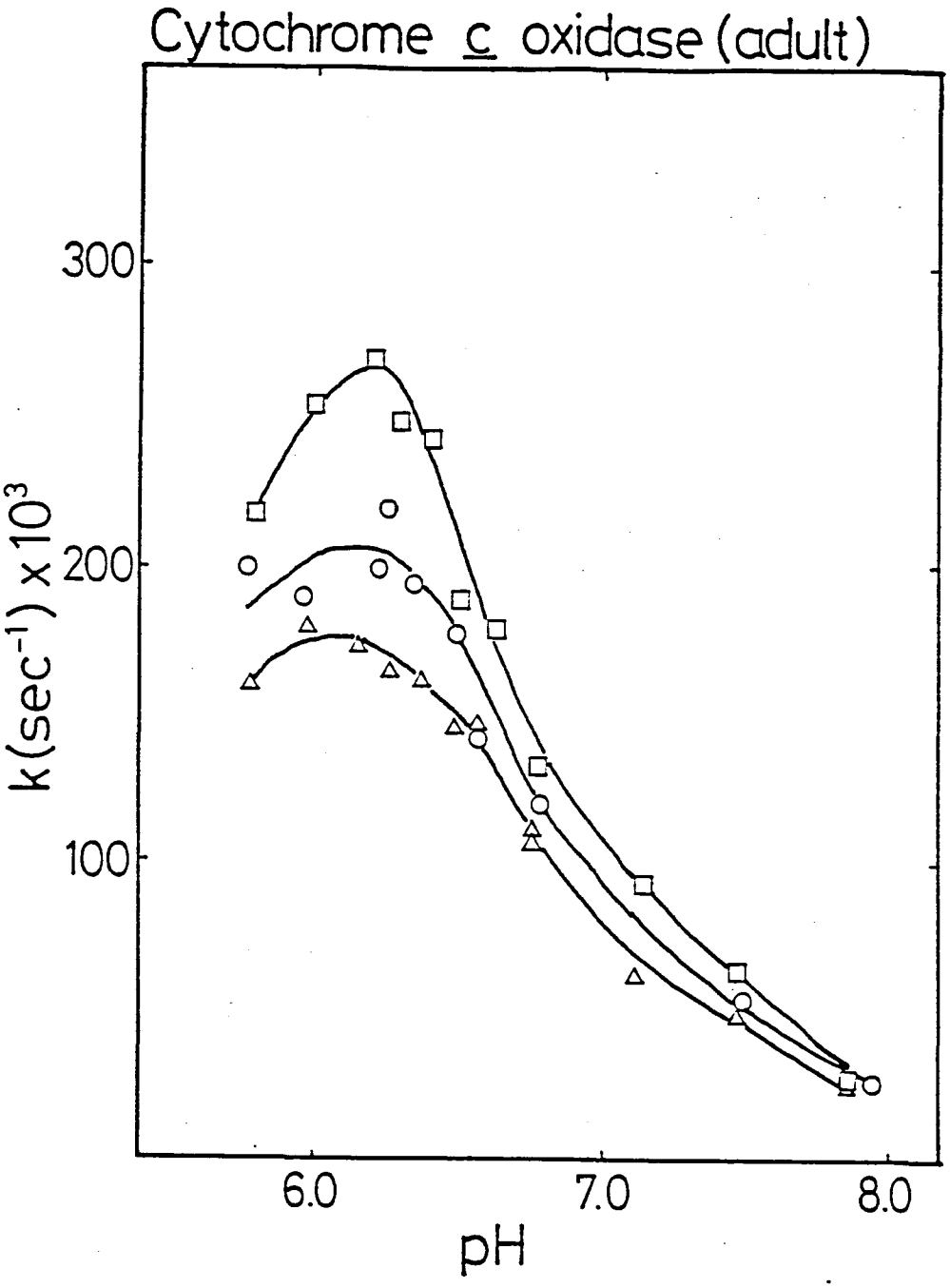


Fig. II-8. Effect of pH on the oxidation rates of horse heart ferrocytochrome c and larval- and adult-type ferrocytochromes c of the housefly with cytochrome c oxidase preparation from adult houseflies. The reaction mixture contained 50 mM phosphate buffer, 4.0 μM cytochrome c, 3.6 nM oxidase in a total amount of 2.0 ml. The reaction was started by addition of the enzyme solution (volume 10 μl). Cytochrome oxidase activity is expressed as the first order velocity constant (k) ($sec^{-1}$). o—o, horse heart cytochrome c; □—□, larval-type cytochrome c; △—△, adult-type cytochrome c.

cytochrome c. It is interesting that some difference was observed in the reactivity between two isocytochromes c with the cytochrome oxidase preparation in spite of only six amino acid difference between them (shown in section III.1). This difference in the reactivity of the two isocytochromes c with the oxidase does not seem to be of physiological importance for the following reasons: (i) Adult cytochrome c oxidase reacts with adult-type cytochrome c more slowly than with larval-type cytochrome c; (ii) the reaction is not thought to be the rate-limiting step in the electron transfer process; (iii) larval-type cytochrome c does not occur in the fruit fly or in the flesh fly (section II.2). Further investigation will be required to make clear whether or not the difference in reactivity with cytochrome c oxidase between two isocytochromes c is reflected on the physiological difference between larvae and adults.

## 2. CYTOCHROMES c OF FRUIT FLY AND FLESH FLY

### 1) MATERIALS AND METHODS

#### Materials

The fruit fly *Drosophila melanogaster* was reared at 25°C and stored at -20°C until use. The flesh fly *Boettcherisca peregrina* was obtained from Japan Insecticide MFG. Co. and stored at -20°C until use.

#### Determination of Cytochrome c Content and Disc Electrophoresis

Cytochrome c contents in individual insects were determined by the method previously described [15]. About 0.2 - 0.5 g of the fruit fly at various stages and of the flesh fly at larval and adult stages were homogenized in 0.5% $Al_2(SO_4)_3$, pH 4.5, and each homogenate was kept at room temperature for 2 h. The homogenate thus treated was centrifuged at 20,000 x g for 15 min, the resulting supernatant was neutralized with 1 M $NH_4OH$ and recentrifuged at 20,000 x g for 15 min. The supernatant thus obtained was charged on a small Amberlite CG-50 column and cytochrome c adsorbed on the column was eluted with 20 mM sodium phosphate buffer, pH 8.0 containing 500 mM NaCl. The volume of the resulting eluate was adjusted to 1.0 ml and the amount of cytochrome c in this eluate was determined by the absorbance at 550 nm after the cytochrome was fully reduced with $Na_2S_2O_4$. The $\varepsilon_{mM}$ at 550 nm of cytochrome c was assumed to be 27.7 [24].

The eluate obtained above was dialyzed against 20 mM

ammonium phosphate buffer containing 40% sucrose and subjected to gel electrophoresis at a running pH of 6.6 as previously described [14]. After electrophoresis, cytochrome c in the gel was detected by heme staining [19].

Purification of larval and adult cytochromes c of the fruit fly and the flesh fly

To purify larval and adult cytochromes c of the fruit fly and the flesh fly, the insects were separately homogenized with 0.5% $Al_2(SO_4)_3$ [23]. After the homogenate was allowed to stand for 2 h, it was centrifuged at 20,000 x g for 20 min, pH of the resulting supernatant was adjusted to 8.0 with addition of $NH_4OH$, and then the solution obtained was centrifuged at 20,000 x g for 20 min. The supernatant thus obtained was charged on an Amberlite CG-50 column equilibrated with 20 mM phosphate buffer (pH 8.0) and cytochrome c was eluted with 20 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl. The eluate obtained was 90% saturated with ammonium sulphate and centrifuged at 20,000 x g for 20 min. The resulting supernatant was charged on a DEAE-cellulose column which had been equilibrated with 90% saturated ammonium sulphate solution. The cytochrome adsorbed on the column was eluted with 20 mM ammonium phosphate buffer (pH 6.8) and the resulting eluate which contained cytochrome c was dialyzed against the same buffer as used for the above elution. The dialyzed cytochrome c solution was charged on an Amberlite CG-50 column and cytochrome c adsorbed on the column was eluted with a linear gradient from 20 mM to 300 mM ammonium phosphate buffer (pH 6.8). The red-coloured eluate thus obtained was used

as the purified cytochrome c preparation.

Amino Acid Analysis

The amino acid composition of proteins and peptides was analyzed with a high sensitivity amino acid analyzer (IRICA Instruments, Inc., model A-3300, Kyoto, Japan) after the samples were hydrolyzed with 6 N HCl for 24 h or 72 h at 110°C in evacuated sealed tubes.

Tryptic Digestion and Peptide Mapping

One mg each of the larval and adult cytochrome c preparations of the fruit fly and the flesh fly was digested in 0.1 M $NH_4HCO_3$ with 20 μg TPCK-trypsin overnight at 40°C.

Peptide mapping of fruit fly cytochrome c was performed as described by Ando et al. [34] and the resulting peptide spots were detected by 0.002% fluorescamine-acetone. The peptide spots appeared were separately extracted by 0.5 N acetic acid overnight and subjected to amino acid analysis.

Peptide mapping of flesh fly cytochrome c was performed by high performance liquid chromatography (HPLC) (IRICA Chromatic LC-300 PUS System, Kyoto) in 0.1% trifluoroacetic acid with a linear gradient from 0 to 70% acetonitrile on an octadecylsilane column (IRICA RP-18, 4 x 250 mm, 0.8 ml/min) at 60°C according to the methods previously described [35]. Each peptide fraction was dried and subjected to amino acid analysis.

## 2) RESULTS

Fruit fly cytochrome c

Variation of cytochrome c level during the development of the fruit fly is shown in Fig. II-9. On adult emergence, the amount of cytochrome c was increased approximately 6-fold as compared with that at the late pupal stage.

The electrophoretic patterns of fruit fly cytochrome c during metamorphosis are shown in Fig. II-10. Only one band was detected in the gel on heme staining at any developmental stages. This shows that there are no other species of cytochrome c that show different electrophoretic mobility in the fruit fly.

The amino acid composition of cytochrome c purified from larvae and adults are shown in Table II-IV. No difference was observed in the composition of cytochrome c between larvae and adults. The peptide map (Fig. II-11) also showed the same patterns between the larval and adult cytochrome c preparations. The amino acid compositions of all the spots were analyzed (data not shown). They showed no difference between the larval and adult protein preparations and this was explicable by the sequence described in section III.2.

Flesh fly cytochrome c

The amount of cytochrome c in larvae of the flesh fly was 1.02 μg per insect and 23.1 μg per g wet weight, and that of adults was 10.4 μg per insect and 325 μg per g wet weight. The amount of cytochrome c was increased approximately 10-fold on adult emergence.

The electrophoretic patterns of cytochrome c separately purified from larvae and adults of the flesh fly are shown in

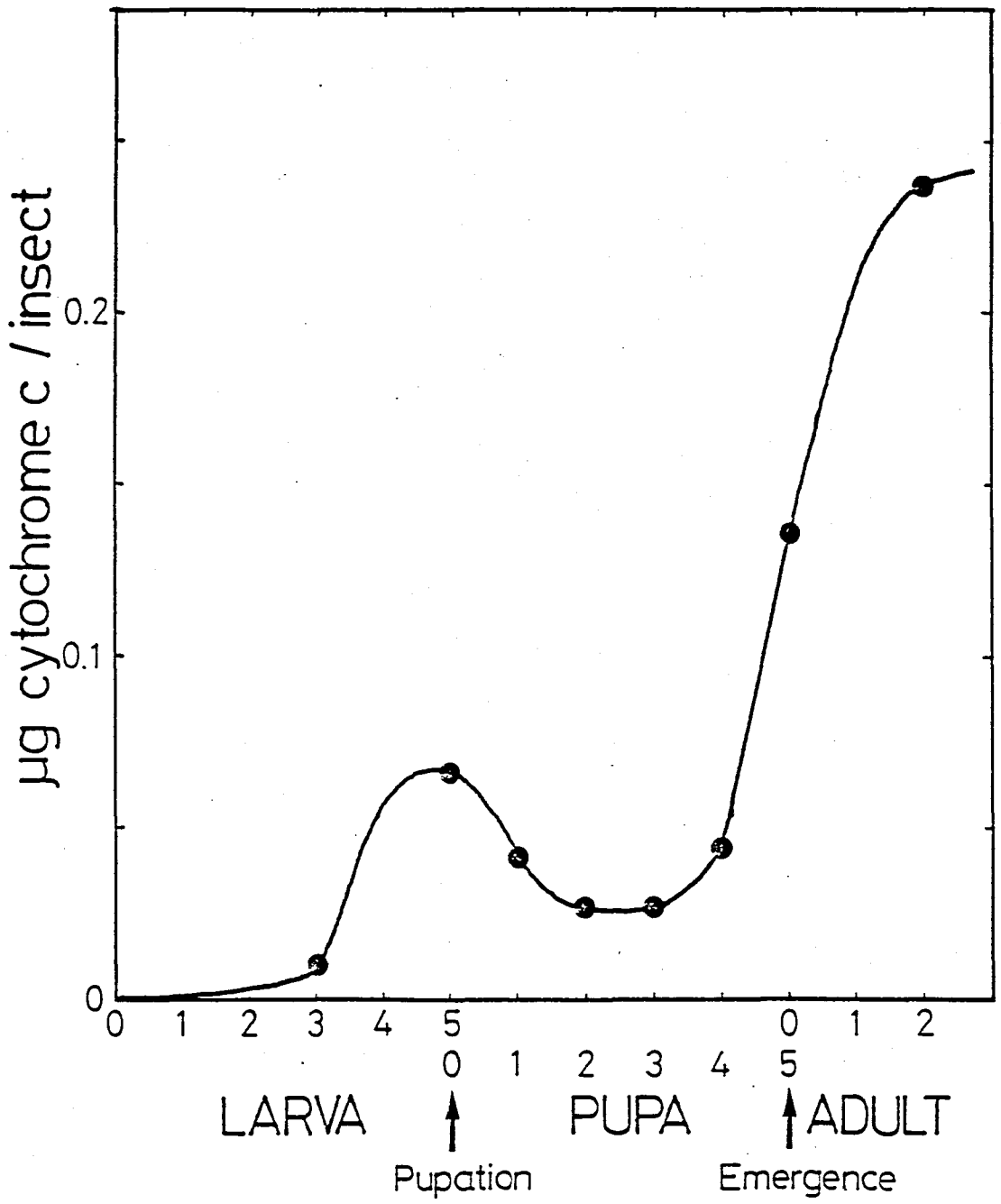


Fig. II-9. Variation in the cytochrome c content during the metamorphosis of the fruit fly. The amount of cytochrome c was determined by the absorbance at 550 nm ( $\varepsilon_{mM}$ at 550 nm = 27.7) after it was reduced by dithionite. About a thousand insects were used in each experiment.

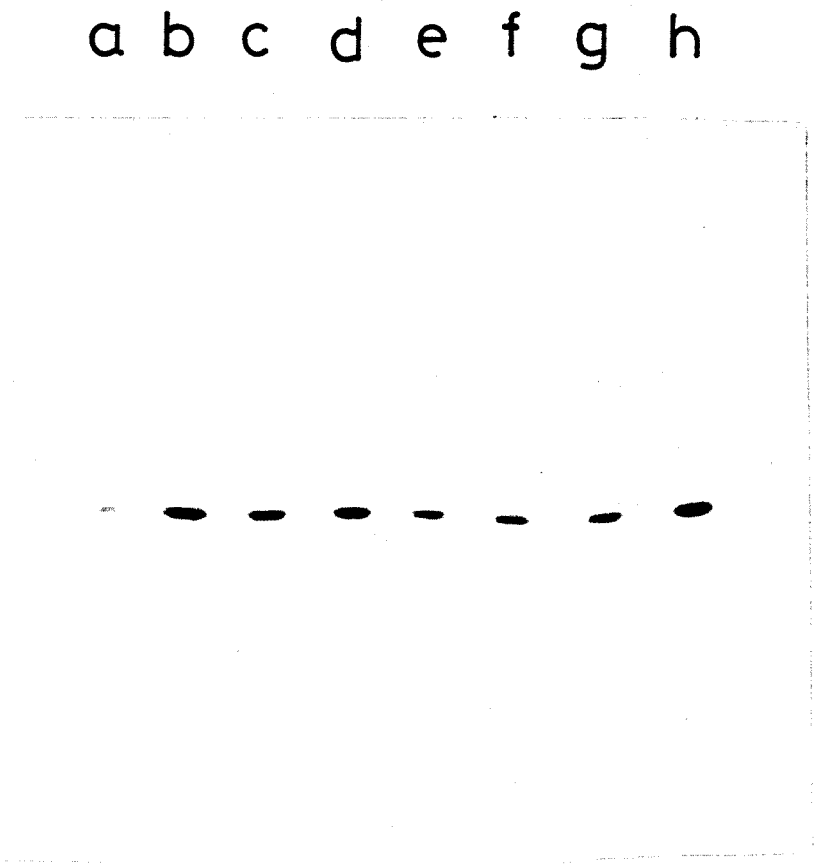


Fig. II-10. Electrophoretic patterns of cytochromes c during development of the fruit fly. Gel was stained with heme staining. The samples containing about 2 μg cytochrome c were applied to each lane. Lane a, 2nd inster larvae; lane b, 3rd inster larvae; lane c, pupae (1 day after the pupation); lane d, pupae (2 days); lane e, pupae (3 days); lane f, pupae (4 days); lane g, adults (just after the emergence); lane h, adults (2 days after the emergence.

| | Sequence | Cyt c (larva) | Cyt c (adult) |
|---|---|---|---|
| Cys | 2 | n.d.* | n.d.* |
| Asp | 9 | 9.22 | 8.86 |
| Thr | 7 | 7.49 | 7.04 |
| Ser | 1 | 1.36 | 1.12 |
| Glu | 9 | 10.3 | 9.90 |
| Pro | 5 | 4.79 | 4.64 |
| Gly | 14 | 14.6 | 14.0 |
| Ala | 11 | 10.3 | 9.72 |
| Val | 5 | 4.46 | 4.49 |
| Met | 1 | 0.77 | 0.79 |
| Ile | 5 | 4.46 | 4.49 |
| Leu | 8 | 7.72 | 7.48 |
| Tyr | 4 | 4.17 | 4.33 |
| Phe | 4 | 4.36 | 4.34 |
| Lys | 15 | 15.1 | 15.9 |
| His | 3 | 2.84 | 3.00 |
| Arg | 3 | 2.57 | 2.48 |
| Trp | 1 | n.d.* | n.d.* |
| Total | 107 | | |

Table II-IV Amino acid compositions of larval and adult cytochrome c of the fruit fly. The amino acid analyses were performed on 24-h hydrolysates. The composition based on the sequence is also included. * Not determined.

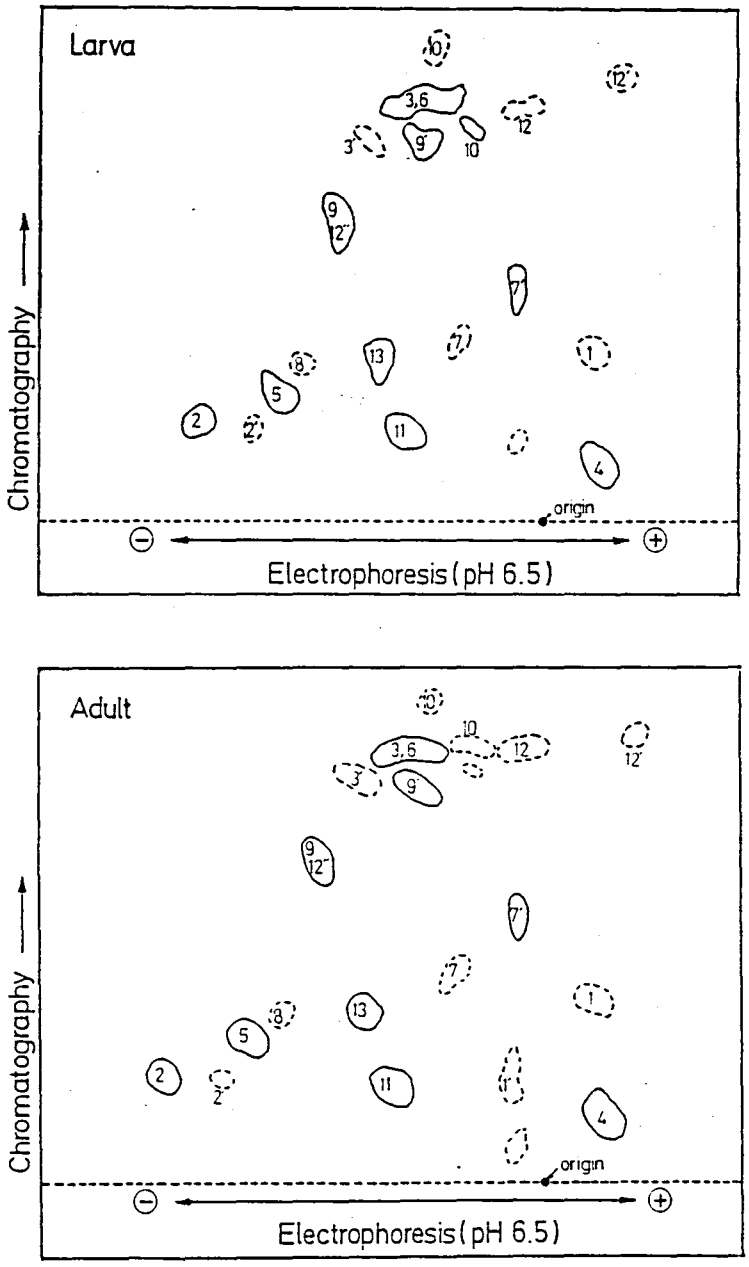


Fig. II-11. Peptide maps of the tryptic peptides derived from larval and adult cytochromes c of the fruit fly. Electrophoresis was performed at pH 6.5 in the buffer pyridine / acetic acid / water (10 : 0.4 : 180), at 2500 V for 45 min. Descending chromatography was performed for 16 h using the solvent n-butanol / pyridine / acetic acid / water (15 : 10 : 3 : 12). The spots were detected by fluorescence after they had been stained with fluorescamine. Numbered spots were cut out, extracted with 0.5 N acetic acid overnight and subjected to amino acid analysis. The same numbered spots between larval and adult contain the peptides with same composition. The spot numbered with prime contained a part of the peptide from the spot with the same number.

Fig. II-12. Only one band was detected in the gel on heme staining with the cytochrome c preparations of both larval and adult stages. This shows that there are no other species of cytochrome c that show different electrophoretic mobility in the flesh fly.

The amino acid composition of cytochrome c purified from larvae and adults are shown in Table II-V. No difference was observed in the composition of cytochrome c between larvae and adults except that methionine was not detected in the larval protein. The peptide map by HPLC (Fig. II-13) also showed the same elution patterns between the larval and adult cytochrome c preparations. The amino acid compositions of all the fractions obtained by the peptide mapping were analyzed (data not shown). They showed no difference between the larval and adult protein preparations and this is explicable by the sequence described in section III.2.

## 3) DISCUSSION

A dramatic increase of the cytochrome c content takes place at the adult emergence in both fruit fly and flesh fly. Similar results have been obtained with other insects such as the moth Platysamia cecropia [36] and Samia cynthia [37], the butterfly Pieris brassicae [38], the flies Phormia regina [39], Lucilia cuprina [23] and Musca domestica [14,15] (in section II.1.b), the honeybee [40,41] (in section II.3) and the colorado beetle [42]. The increase in cytochrome c reflects the increase of

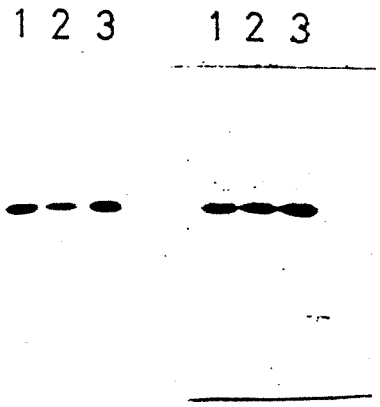


Fig. II-12. Electrophoretic patterns of cytochrome c separately purified from larvae and adults of the flesh fly. (A) Heme staining with benzidine-$H_2O_2$; (B) Coomassie blue staining. Lane 1, larval cytochrome c; lane 2, the mixture of larval and adult cytochromes c; lane 3, adult cytochrome c.

| | Sequence | Cyt c (larva) | Cyt c (adult) |
|---|---|---|---|
| Cys | 2 | n.d.* | n.d.* |
| Asp | 9 | 8.79 | 8.89 |
| Thr | 7 | 6.77 | 6.94 |
| Ser | 1 | 1.42 | 1.33 |
| Glu | 9 | 10.6 | 10.1 |
| Pro | 6 | 5.57 | 5.29 |
| Gly | 14 | 14.3 | 14.0 |
| Ala | 10 | 9.59 | 9.44 |
| Val | 5 | 4.47 | 4.67 |
| Met | 1 | 0.00 | 0.83 |
| Ile | 5 | 4.84 | 4.79 |
| Leu | 7 | 6.77 | 6.76 |
| Tyr | 4 | 3.75 | 3.91 |
| Phe | 5 | 5.40 | 5.22 |
| Lys | 15 | 14.9 | 15.2 |
| His | 3 | 3.40 | 3.38 |
| Arg | 3 | 2.86 | 2.83 |
| Trp | 1 | n.d.* | n.d.* |
| Total | 107 | | |

Table II-V Amino acid compositions of larval and adult cytochrome c of the flesh fly. The amino acid analyses were performed on 24-h hydrolysates. The composition based on the sequence is also included. * Not determined.

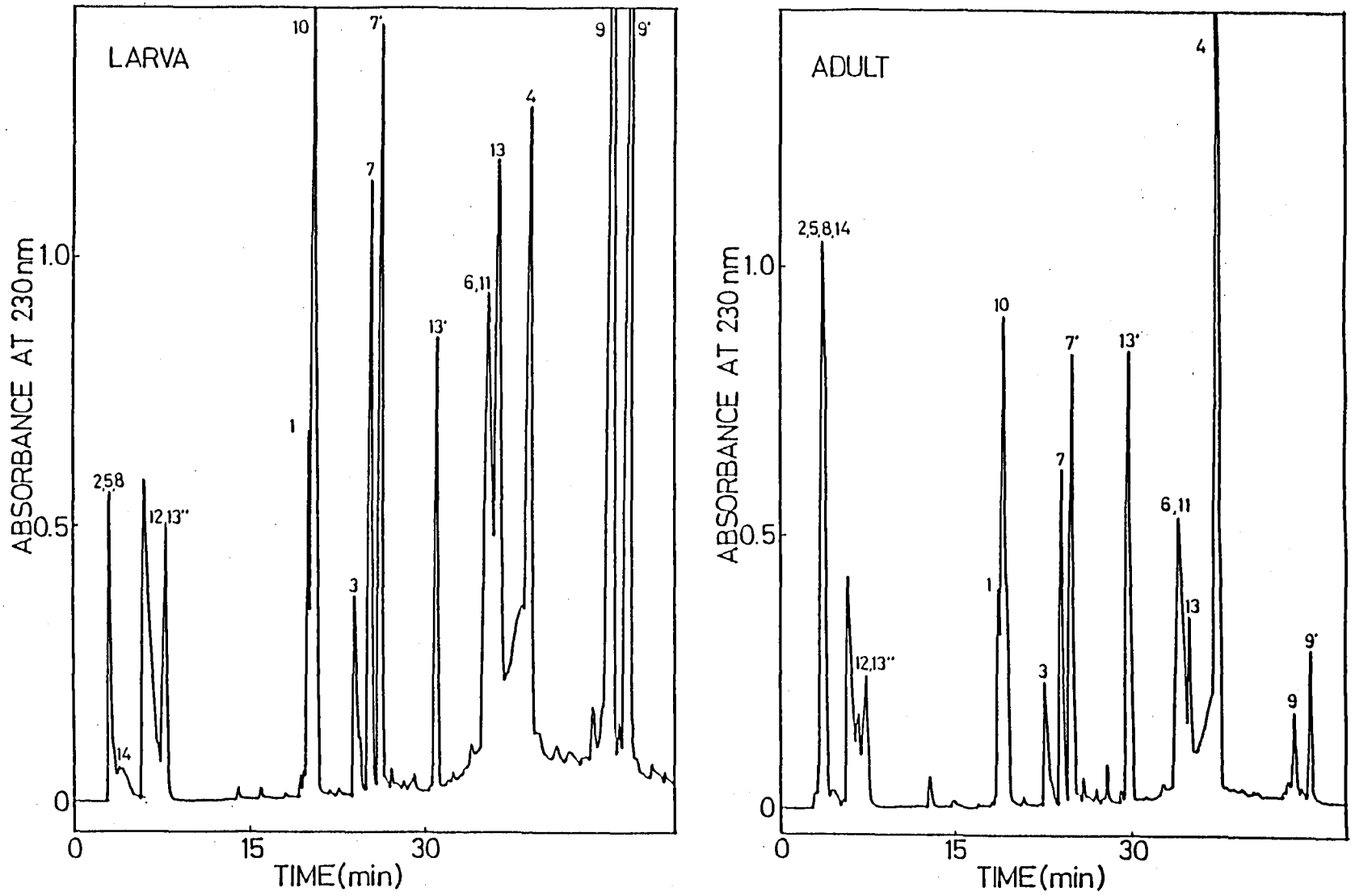


Fig. II-13. Peptide mappings of flesh fly cytochromes c purified from larvae and adults. Peptide mapping was performed by HPLC as described in "MATERIALS AND METHODS".

mitochondria during flight muscle development.

In contrast with the housefly, isocytochromes c were not detected in the fruit fly and the flesh fly as judged from the results of electrophoresis, amino acid analysis and peptide mapping. This will suggest that the cytochrome c species which corresponds to the larval-type cytochrome c of the housefly may not be genetically expressed in the fruit fly and the flesh fly. It is interesting that the change of cytochrome c content during the development of the fruit fly (Fig. II-9) was similar to that of the housefly (Fig. II-3). This suggests that larval-type cytochrome c of the housefly can be replaced with adult-type protein and that there is no difference between them in their physiological functions. This might be further evidence for that larval-type cytochrome c is constitutive protein and adult-type cytochrome c is adaptive protein. The sequence studies of fruit fly and flesh fly cytochromes c will be described in section III.2 and the evolutional appearance of larval-type cytochrome c in insect will be further discussed in section IV.2.

## 3. CYTOCHROME c OF HONEYBEE

### 1) MATERIALS AND METHODS

#### Materials

The honeybee, Apis. mellifera was obtained from a local beekeeper in Wakayama Prefecture. The honeybees were frozen with liquid nitrogen and stored at -20°C until use.

#### Determination of Cytochrome c Content by Disc Electrophoresis

Cytochrome c contents in individual insects were determined by the method previously described [15]. Larvae, pupae, and adults (0.2 - 9 g) were separately homogenized in 0.5% $Al_2(SO_4)_3$, pH 4.5, and each homogenate was kept at room temperature for 2 h. The homogenate thus treated was centrifuged at 20,000 x g for 15 min, the resulting supernatant was neutralized with 1 M $NH_4OH$ and recentrifuged at 20,000 x g for 15 min. The supernatant thus obtained was charged on a small Amberlite CG-50 column and cytochrome c adsorbed on the column was eluted with 20 mM sodium phosphate buffer, pH 8.0 containing 500 mM NaCl. The volume of the resulting eluate was adjusted to 1.0 ml and the amount of cytochrome c in this eluate was determined by the absorbance at 550 nm after the cytochrome c was fully reduced with $Na_2S_2O_4$. The $e_{mM}$ at 550 nm of cytochrome c was assumed to be 27.7 [24].

The eluate obtained above was dialyzed against 100 mM $NH_4HCO_3$, lyophilized and subjected to gel electrophoresis at a running pH of 6.6 as previously described [14]. After

electrophoresis, the proteins in the gel were stained by Coomassie brilliant blue R-250 and cytochrome c in the gel was detected by heme staining [19].

Peptide Mapping by HPLC

For the peptide mapping in a small scale, cytochrome c was recovered from the gel after the electrophoresis; cytochrome c band in the gel was cut out before staining. The gel fraction cut out was homogenized with 100 mM $NH_4HCO_3$ and the homogenate thus obtained was centrifuged at 20,000 x g for 15 min to discard gel fragments, and the precipitated gel fractions were reextracted for 1 h. Then, the combined supernatant was dialyzed against 100 mM $NH_4OH$ overnight, and then lyophilized. The lyophilized powder was dissolved in 0.1 ml of 100 mM $NH_4HCO_3$ containing trypsin (5 μg / ml) and tryptic digestion of the cytochrome c preparation was performed at 40°C overnight.

Peptide mapping was performed by high performance liquid chromatography (IRICA Chromatic LC-300 PUS System, Kyoto) in 0.1% trifluoroacetic acid with a linear gradient from 0 to 70% acetonitrile on an octadecylsilane column (IRICA RP-18, 4 x 250 mm, 0.8 ml/min) at 60°C according to the methods previously described [35].

Amino Acid Analysis

Amino acid compositions of honeybee cytochrome c were determined with an amino acid analyzer (IRICA model A-3300) after the samples were hydrolyzed with 6 N HCl for 24 h at 110°C in evacuated sealed tubes.

## 2) RESULTS

Table II-VI shows the amounts of cytochrome c in larvae, pupae and adults of worker and drone, and in a queen adult. The amount of cytochrome c was very low at the larval and pupal stages especially of worker, while that in adult was more than 30-fold as much as that in pupae. It was also shown that the cytochrome c content in drone was 3-fold higher than that in worker through all the developmental stages.

The electrophoretic patterns of cytochrome c obtained from the three developmental stages of the honeybee are shown in Fig. II-14. Only one band was detected in the gel on heme staining at any developmental stages of any castes. This shows that there are no other species of cytochrome c that show different electrophoretic mobility in the honeybee. Cytochrome c was extracted from the polyacrylamide gel after the electrophoresis. The peptide mapping by HPLC of the tryptic digests of cytochrome c extracted from the gel showed no difference in the elution profile between the any cytochrome c preparations (Fig. II-15). This result will reinforce above finding that there are no isocytochromes c in the honeybee.

The amino acid compositions of cytochrome c preparations obtained from worker larvae and adults are shown in Table II-VII. No difference was observed in the composition between the cytochrome preparations from the larvae and adults, except that the value of tyrosine for the larval protein was lower than that

| | Larva | Pupa | Adult |
|---|---|---|---|
| Worker | 0.30 (2.4) | 0.33 (2.8) | 12.5 (131) |
| Drone | 0.93 (4.5) | 1.13 (6.0) | 33.2 (172) |
| Queen | n.d.* | n.d.* | 33.4 (187) |

Table II-VI. Variation in the cytochrome c content during metamorphosis of the honeybee. The values are expressed as μg / insect, and those in parentheses are μg / g wet body weight.
* Not determined.

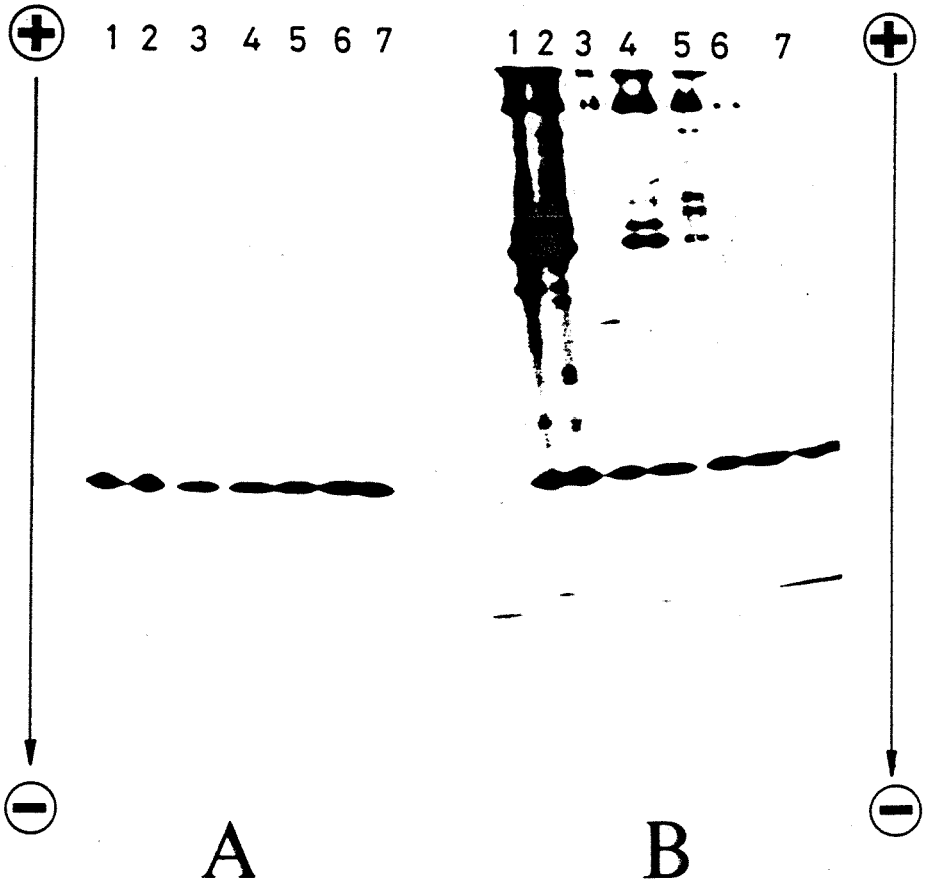


Fig. II-14. Electrophoretic patterns of honeybee cytochrome c. (A) Heme staining with benzidine-$H_2O_2$; (B) Coomassie blue staining. Samples were from larvae (lane 1), pupae (lane 2) and adults (lane 3) of worker, and larvae (lane 4), pupae (lane 5) and adults (lane 6) of drone, and adult of queen (lane 7).

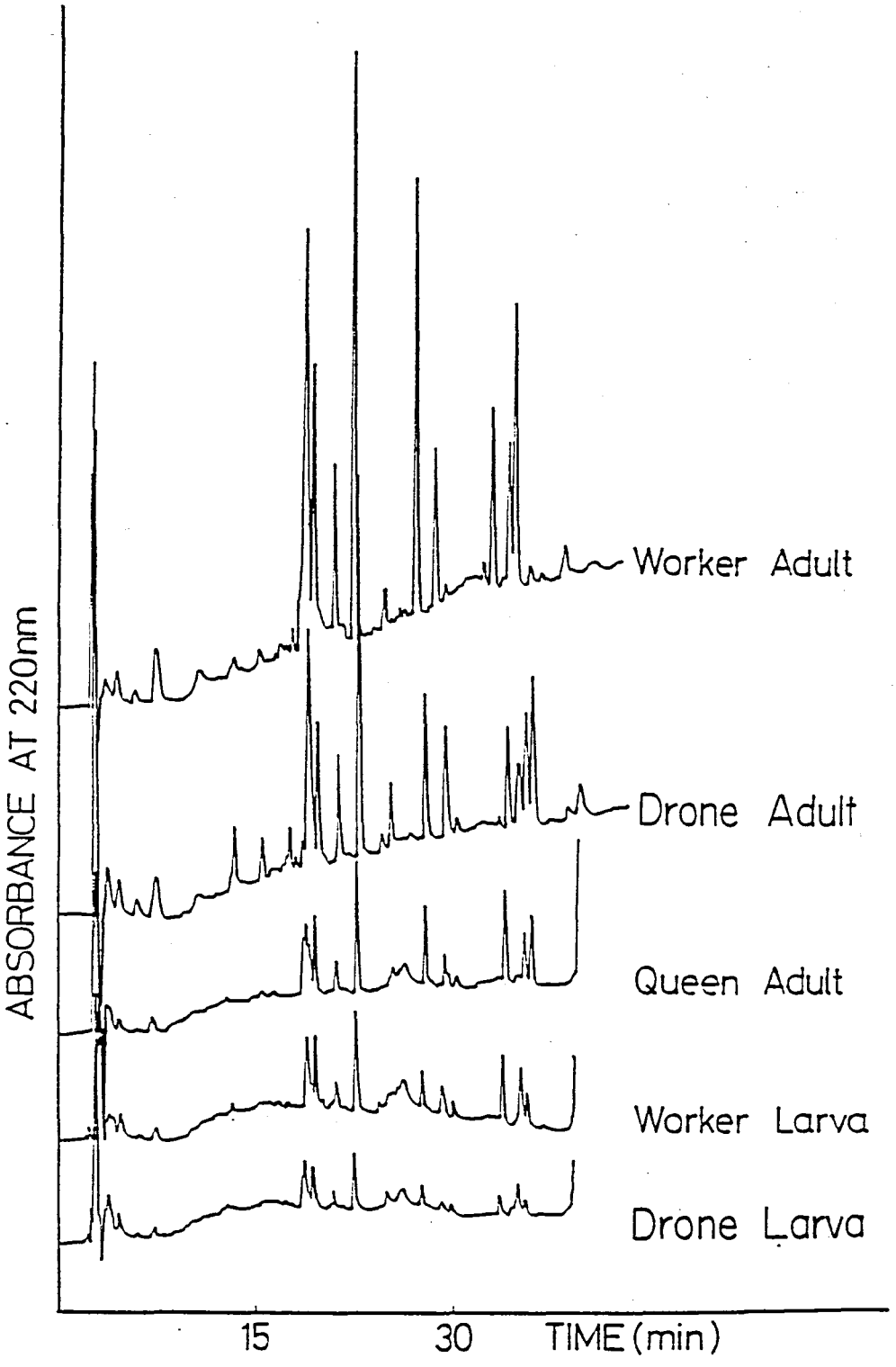


Fig. II-15. Peptide mappings of honeybee cytochrome c by HPLC. Each cytochrome c preparation was extracted from the gel after polyacrylamide gel electrophoresis as shown in Fig. II-14.

| | Sequence (this work) | | Cyt c (larva) | Cyt c (adult) | Cm-cyt c (adult) |
|---|---|---|---|---|---|
| Cmc | 2 | | - | - | 1.91 |
| Asp | 3 | 7 | 7.06 | 6.79 | 7.02 |
| Asn | 4 | | | | |
| Thr | 6 | | 5.74 | 5.78 | 5.87 |
| Ser | 3 | | 2.99 | 2.88 | 2.83 |
| Glu | 7 | 12 | 13.5 | 13.3 | 13.5 |
| Gln | 5 | | | | |
| Pro | 7 | | 6.24 | 6.58 | 6.73 |
| Gly | 14 | | 13.5 | 13.7 | 13.8 |
| Ala | 8 | | 7.44 | 6.96 | 7.48 |
| Val | 4 | | 3.18 | 3.01 | 3.52 |
| Met | 1 | | 0.73 | 0.76 | 0.84 |
| Ile | 7 | | 6.39 | 6.33 | 6.69 |
| Leu | 5 | | 4.67 | 4.52 | 4.67 |
| Tyr | 7 | | 3.79 | 6.01 | 6.39 |
| Phe | 3 | | 2.91 | 2.86 | 2.92 |
| Lys | 16 | | 16.0 | 17.2 | 16.3 |
| His | 2 | | 2.12 | 2.09 | 2.09 |
| Arg | 2 | | 2.06 | 1.80 | 2.01 |
| Trp | 1 | | n.d.* | n.d.* | n.d.* |
| Total | 107 | | | | |

Table II-VII. Amino acid compositions of cytochrome c preparations from worker larvae and adults. The amino acid analyses were performed on 24-h hydrolysates. The composition based on the sequence is also included. * Not determined.

for the adult protein. This seemed attributable to the underestimation due to the destruction of the amino acid during the acid hydrolysis. The compositions obtained above were in good agreement with that obtained from the sequence as described in section III.3.

## 3)DISCUSSION

The increase in the cytochrome c level on adult emergence has been reported in several insects [14,23,36-42]. As shown in Table II-VI, the cytochrome c content in worker adults was approximately 40 times higher than that in worker pupae. The increase in the cytochrome c content on adult emergence is probably due to the development of mitochondria in the flight muscles [43]. It has been already reported [44] that the cytochrome c level in queen is higher than that in worker and this difference may be related to the caste determination. In the present study, it has been shown that the cytochrome c content in drone is also two or three times as much as that in worker at all developmental stages.

We have already reported that isocytochromes c (larval-type and adult-type cytochromes c) occur in the housefly, Musca domestica [13-15]. The present study shows that isocytochromes c do not occur in the honeybee, Apis mellifera. As shown in Fig. II-14, no other species of cytochrome c have been found which are separatable on electrophoresis. This is consistent with the results obtained by Eder et al. [45]. However, the possibility

remains that electrophoretically indistinguishable isocytochromes c may exist. The amino acid compositions of cytochrome c obtained from worker larvae and adults show no difference between them (Table II-VII). The peptide mapping by HPLC (Fig. II-15) of tryptic digests of the cytochrome c preparations from the honeybees in different castes and different metamorphic stages show the same elution profile. Therefore, we conclude that isocytochromes c do not exist in the honeybee.

# III. SEQUENCE STUDIES OF INSECT CYTOCHROMES *C*

# 1. SEQUENCE STUDIES OF LARVAL- AND ADULT-TYPE CYTOCHROMES C OF THE HOUSEFLY

## 1) MATERIALS AND METHODS

### Materials

The enzymes and chemicals used for the sequence determination in the present experiment were described in the previous papers [46,47]. Larval- and adult-type cytochromes c of the housefly were separately purified from larvae and adults respectively as described in section II.1.d. S-Carboxymethyl-cytochrome c was prepared by the method of Crestfield et al. [48] after removal of heme according to the method of Fontana et al. [49].

### Amino Acid Analysis and Identification of PTH-Amino Acids

The amino acid composition of proteins and peptides were analyzed with a high sensivity amino acid analyzer (IRICA Instruments, Inc., model A-3300, Kyoto, Japan) after the samples were hydrolyzed with 6 N HCl for 24 h or 72 h at 110°C in an evacuated sealed tubes.

Phenylthiohydantoin derivatives of amino acid (PTH-amino acids) were identified by high performance liquid chromatography (HPLC) (IRICA) [50], and in some cases by thin-layer chromatography (TLC) with the solvent V [51].

N-Terminal and C-Terminal Analysis

The N-terminal sequences of proteins and peptides were determined by manual Edman degradation [52]. The C-terminal residues were determined by carboxypeptidase digestion [53]; peptide or protein was digested by carboxypeptidase A or B for 30 min and 60 min in 0.1 M Tris-HCl buffer (pH 8.0) at 40°C, and the amino acids released were determined with amino acid analyzer.

Tryptic Digestion and Peptide Map

Two mg each of CM-proteins derived from larval- and adult-type cytochromes c was digested in 0.1 M $NH_4HCO_3$ with 20 μg trypsin (Worthington Biochemical Corp., U.S.A.) overnight at 40°C. Peptide mapping was performed as described by Ando et al. [34] and the resulting peptide spots were detected by 0.002% fluorescamine-acetone. The peptide spots appeared were separately extracted by 0.5 M acetic acid overnight, and the extracts were subjected to amino acid analysis or sequence study.

Staphylococcal Protease and Chymotryptic Digestion

Three mg each of CM-cytochromes c was digested in 0.1 M $NH_4HCO_3$ with 30 μg Staphylococcus aureus V8 protease (Miles Laboratories, U.K.) at 40°C for 48h. Chymotryptic digestion was performed by incubating 1 mg each of intact cytochromes c with 10 μg chymotrypsin in 0.1 M $NH_4HCO_3$ at 40°C for 4 h.

Peptides thus obtained were separated by HPLC in 0.1% trifluoroacetic acid with a linear gradient from 0 to 70% acetonitrile on an octadecylsilane column (IRICA RP-18, 4 x 250 mm, 0.8 ml/min) at 60°C in similar ways to those described

previously [35]. Some peptides were further purified by high-voltage paper electrophoresis at pH 6.5 or 3.6 [54].

Nomenclature

LT-, LS- and LC refer to tryptic peptides, staphylococcal protease peptides and chymotryptic peptides from larval-type cytochrome c, respectively. AT-, AS- and AC- refer to tryptic peptides, staphylococcal protease peptides and chymotryptic peptides from adult-type cytochrome c, respectively.

## 2) RESULTS

The amino acid compositions of larval- and adult-type cytochromes c were already shown in Table II-I. The compositions were in good agreement with those obtained from the sequence as described below.

The N-terminal sequences of larval- and adult-type cytochromes c were determined by manual Edman degradations to be Gly-Val-Pro-Ala-Gly-Asp-Val-Glu-Lys-Gly-Lys-Lys-Leu-Phe-Val-Gln-Arg-(Cys)-Ala-Gln and Gly-Val-Pro-Ala-Gly-Asp-Val-Glu-Lys-Gly-Lys-Lys-Ile-Phe-Val-Gln-Arg-(Cys)-Ala-Gln, respectively.

Alanine (0.83 mol / mol protein) was released from larval-type cytochrome c in 30 min digestion by carboxypeptidase A, and alanine (1.06), threonine (0.39) and serine (0.14) were released in 60 min digestion. Lysine (0.79) was released from adult-type cytochrome c in 30 min digestion by carboxypeptidase B. These were consistent with the C-terminal sequences of larval-type and

adult-type cytochromes c (Fig. III-3).

Peptide maps of tryptic digests obtained from larval- and adult-type cytochromes c are shown in Fig. III-1, and amino acid compositions of tryptic peptides are summarized in Table III-I. Tryptic peptides, extracted from peptide map, were subjected to the sequence studies as shown below. As N-terminal residues of the peptides obtained here were partially blocked by fluorescamine, the recovery of PTH-amino acids was low. As the peptides LT-11 and AT-11 were hydrophobic, their yield was low and PTH-amino acid derived from them could not be detected.

To obtain overlaps between tryptic peptides, cytochromes c were digested with chymotrypsin and staphylococcal protease. Peptides were separated by HPLC (Fig. III-2). Three (LC-1, LC-2, LC-3) of the peptides obtained from chymotryptic digests for larval-type cytochrome c and six (AC-1-AC-6) of those for adult-type cytochrome c were subjected to sequence studies. The peptides LC-1 and LC-3 were further purified by paper electrophoresis at pH 3.6 and the peptide AC-5 was purified by paper electrophoresis at pH 6.5. The amino acid compositions of the peptides obtained are summarized in Table III-II. Tryptophan was released by carboxypeptidase A digestion of the peptides LC-2 and AC-3.

Staphylococcal protease digestion was performed over 48 h. Many heterogeneous cleavages occurred and more peptide fractions were obtained than expected. Seven peptides obtained from digests of the larval protein and eight peptides obtained from digests of the adult protein were subjected to sequence studies. The amino acid compositions of these fractions were summarized in

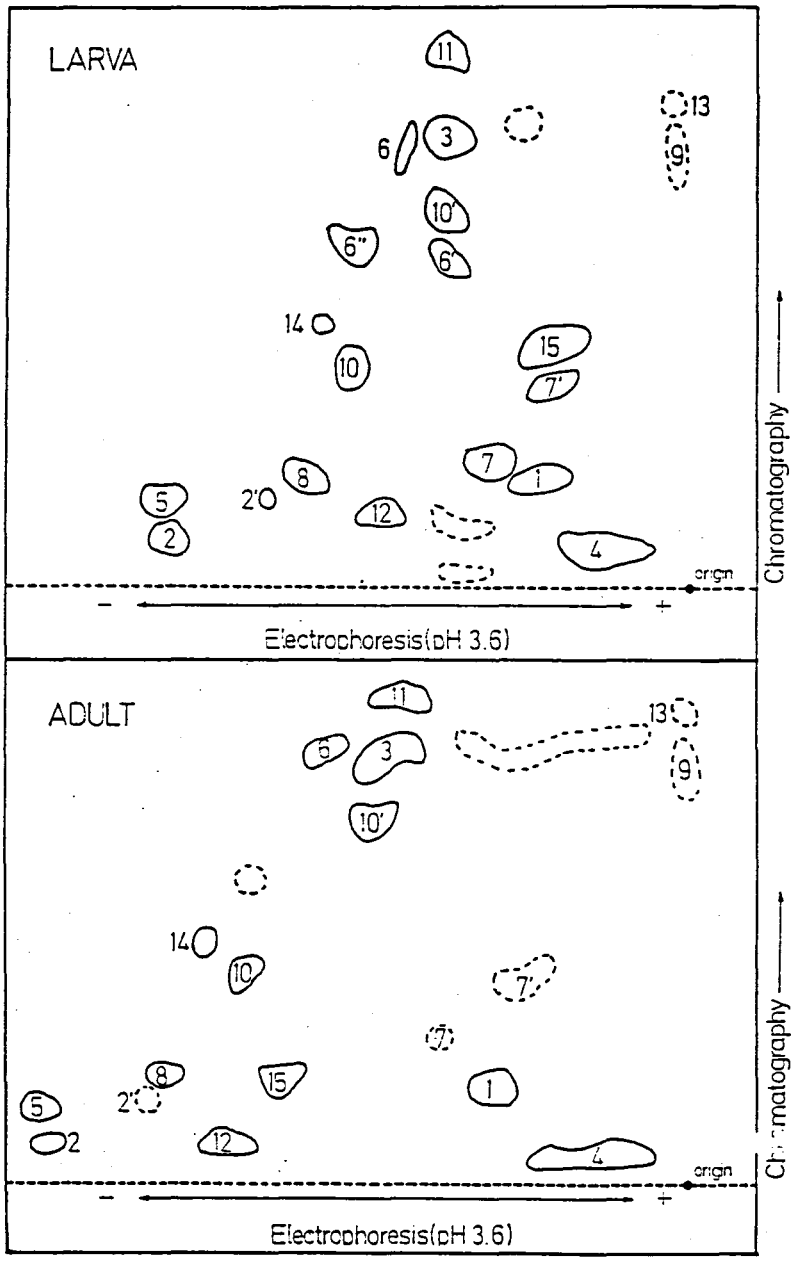


Fig. III-1. Peptide maps of the tryptic peptides derived from larval- and adult-type cytochromes c of the housefly. Electrophoresis was performed at pH 3.6 in the buffer pyridine / acetic acid / water (1 : 10 : 189), at 2500 V for 1 h. Descending chromatography was performed for 16 h using the solvent n-butanol / pyridine / acetic acid / water (15 : 10 : 3 : 12). The spots were detected by fluorescence after they had been stained with fluorescamine. Numbered spots were cut out, extracted with 0.5 N acetic acid overnight and subjected to amino acid analysis. The spot numbered with prime contained a part of the peptide from the spot with the same number.

| | LT-1 | LT-2 | LT-3 | LT-4 | LT-5 | LT-6 | LT-7 | LT-8 |
|---|---|---|---|---|---|---|---|---|
| Cmc | | | | 0.74(2) | | | | |
| Asp | 1.05(1) | | | | | 1.09(1) | 2.12(2) | |
| Thr | | | | 0.93(1) | | | 2.32(2) | |
| Ser | | | | | | | | 0.88(1) |
| Glu | 1.05(1) | | 1.07(1) | 1.92(2) | | | 1.25(1) | |
| Pro | 0.99(1) | | | | | 1.01(1) | | |
| Gly | 1.99(2) | 0.95(1) | | 2.33(2) | | 3.18(3) | 1.83(2) | |
| Ala | 1.04(1) | | | 2.21(2) | | | 3.40(4) | |
| Val | 1.90(2) | | 0.99(1) | 1.02(1) | | 0.93(1) | | |
| Met | | | | | | | | |
| Ile | | | | | | | | |
| Leu | | | 0.95(1) | | | 2.00(2) | | |
| Tyr | | | | | | | 0.22(1) | |
| Phe | | | 1.01(1) | | | 0.96(1) | 0.97(1) | |
| Lys | 0.91(1) | 2.11(2) | | 0.95(1) | 1.07(1) | | 1.60(2) | 1.12(1) |
| His | | | | 0.89(1) | 0.93(1) | 1.01(1) | | |
| Arg | | | 0.98(1) | | | 0.95(1) | | |
| Trp | - | - | - | - | - | - | - | - |
| Yield(%) | 19.6 | 15.8 | 20.3 | 25.5 | 27.2 | 12.9 | 1.8 | 29.7 |
| Residue | 1-9 | 10-12 | 13-17 | 18-29 | 30-31 | 32-42 | 43-57 | 58-59 |

| | LT-9 | LT-10 | LT-10' | LT-11 | LT-12 | LT-13 | LT-14 | LT-15 |
|---|---|---|---|---|---|---|---|---|
| Cmc | | | | | | | | |
| Asp | 2.97(3) | | | | 1.03(1) | 1.09(1) | | |
| Thr | 1.77(2) | 1.10(1) | 0.95(1) | | | | | 1.14(1) |
| Ser | | | | | | | | 0.72(1) |
| Glu | 2.92(3) | | | | | | | |
| Pro | 1.34(1) | 1.12(1) | 1.01(1) | | 1.01(1) | | | |
| Gly | 1.13(1) | 1.25(1) | 1.16(1) | 1.24(1) | 1.06(1) | | | |
| Ala | | | | 0.96(1) | | 1.78(2) | | 2.29(2) |
| Val | | | | | | | | |
| Met | | | | (1) | | | | |
| Ile | 0.96(1) | 0.99(1) | 0.94(1) | 0.83(1) | | 1.01(1) | | |
| Leu | 1.88(2) | | | 1.01(1) | | 1.01(1) | 0.90(1) | |
| Tyr | 0.66(1) | 0.75(1) | 0.55(1) | | | 0.10(1) | | |
| Phe | 0.86(1) | | | 0.87(1) | | | | |
| Lys | 0.92(1) | 1.60(2) | 0.94(1) | 1.10(1) | 0.88(1) | | 1.10(1) | |
| His | | | | | | | | |
| Arg | | | | | 1.02(1) | | | |
| Trp | + | - | - | - | - | - | - | - |
| Yield(%) | 9.2 | 3.2 | 23.6 | 2.9 | 31.1 | 7.9 | 25.2 | 23.6 |
| Residue | 60-76 | 77-83 | 78-83 | 84-90 | 91-95 | 96-101 | 102-103 | 104-107 |

Table III-I(a). Amino acid compositions of tryptic peptides of larval-type Cm-cytochrome c.

| | AT-1 | AT-2 | AT-3 | AT-4 | AT-5 | AT-6 | AT-7 | AT-8 |
|---|---|---|---|---|---|---|---|---|
| Cmc | | | | 0.68(2) | | | | |
| Asp | 1.07(1) | | | | | 1.11(1) | 2.02(2) | |
| Thr | | | | 0.91(1) | | | 1.94(2) | |
| Ser | | | | 0.38 | | | | |
| Glu | 1.03(1) | | 1.13(1) | 1.85(2) | | | 1.06(1) | |
| Pro | 1.03(1) | | | | | 1.04(1) | | |
| Gly | 1.86(2) | 0.93(1) | | 2.40(2) | | 3.05(3) | 2.23(2) | |
| Ala | 1.02(1) | | | 1.81(2) | | | 3.95(4) | 0.98(1) |
| Val | 1.94(2) | | 0.89(1) | 1.05(1) | | 0.88(1) | | |
| Met | | | | | | | | |
| Ile | | | 0.95(1) | | | | | |
| Leu | | | | | | 2.01(2) | | |
| Tyr | | | | | | | 0.45(1) | |
| Phe | | | 0.96(1) | | | 0.97(1) | 0.96(1) | |
| Lys | 0.96(1) | 2.14(2) | | 1.63(1) | 1.10(1) | | 1.81(2) | 1.02(1) |
| His | | | | 1.01(1) | 0.90(1) | 1.04(1) | | |
| Arg | | | 1.07(1) | | | 0.95(1) | | |
| Trp | - | - | - | - | - | - | - | - |
| Yield(%) | 29.7 | 17.9 | 23.8 | 13.2 | 23.9 | 19.3 | 6.9 | 26.0 |
| Residue | 1-9 | 10-12 | 13-17 | 18-29 | 30-31 | 32-42 | 43-57 | 58-59 |

| | AT-9 | AT-10 | AT-10' | AT-11 | AT-12 | AT-13 | AT-14 | AT-15 |
|---|---|---|---|---|---|---|---|---|
| Cmc | | | | | | | | |
| Asp | 3.09(3) | | | | 1.09(1) | 1.17(1) | | |
| Thr | 1.86(2) | 1.00(1) | 0.98(1) | | | | | 1.14(1) |
| Ser | | | | | | | | 0.77(1) |
| Glu | 3.07(3) | | | | 1.17(1) | | | |
| Pro | 1.01(1) | 1.00(1) | 1.05(1) | | 1.01(1) | | | |
| Gly | 1.04(1) | 1.16(1) | 1.12(1) | 1.28(1) | 0.68 | 0.95(1) | | |
| Ala | | | | 0.96(1) | | 0.99(1) | | 1.07(1) |
| Val | | | | | | | | |
| Met | | | | 0.44(1) | | | | |
| Ile | 0.98(1) | 0.97(1) | 0.92(1) | 0.91(1) | | 0.94(1) | | |
| Leu | 2.00(2) | | | 0.95(1) | | 0.95(1) | 0.75(1) | |
| Tyr | 0.73(1) | 0.37(1) | 0.57(1) | | | 0.13(1) | | |
| Phe | 0.89(1) | | | 1.00(1) | | | | |
| Lys | 1.10(1) | 1.75(2) | 0.93(1) | 0.91(1) | 0.80(1) | | 1.25(1) | 1.02(1) |
| His | | | | | | | | |
| Arg | | | | | 0.93(1) | | | |
| Trp | + | - | - | - | - | - | - | - |
| Yield(%) | 13.8 | 3.1 | 24.1 | 4.7 | 8.1 | 7.1 | 1.7 | 35.4 |
| Residue | 60-76 | 77-83 | 78-83 | 84-90 | 91-95 | 96-101 | 102-103 | 104-107 |

Table III-I(b). Amino acid compositions of tryptic peptides of adult-type Cm-cytochrome c. Amino acid analyses were performed on 24-h hydrolysates. Values in parentheses were taken from the sequence. Tryptophan was detected by its fluorescence.

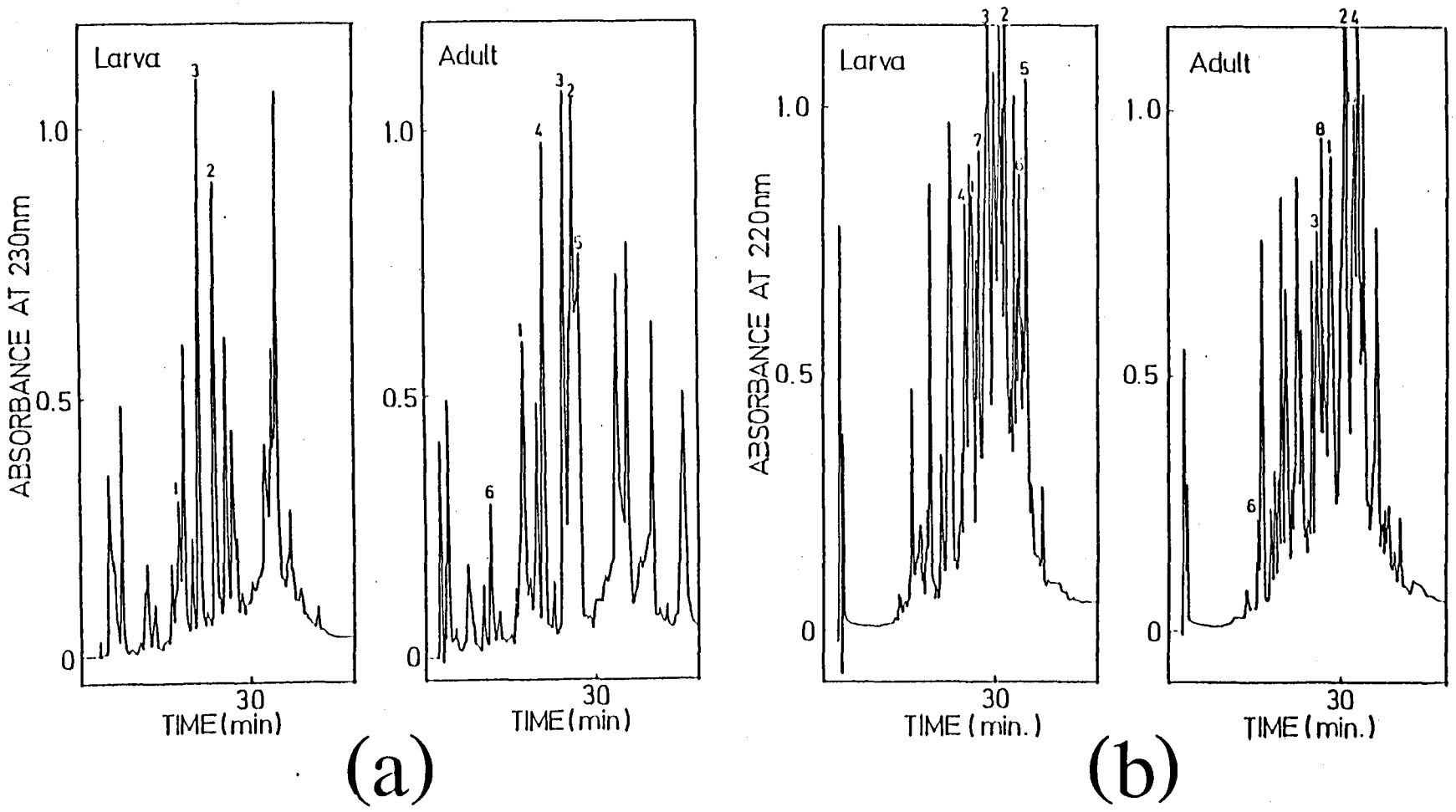


Fig. III-2. (a) Separation of chymotryptic digests of larval- and adult-type cytochromes c. (b) Separation of staphylococcal protease digests of larval- and adult-type cytochromes c. The digests were fractionated by HPLC as described in "MATERIALS AND METHODS".

| | LC-1 | LC-2 | LC-3 |
|---|---|---|---|
| Asp | 0.33 | 2.05(2) | 1.09(1) |
| Thr | 0.75(1) | 1.73(2) | |
| Ser | | 1.04(1) | |
| Glu | 1.15(1) | | 1.95(2) |
| Pro | | | 1.01(1) |
| Gly | 3.13(3) | 1.07(1) | 0.34 |
| Ala | 1.58(2) | 0.85(1) | |
| Val | | | |
| Met | | | |
| Ile | | 0.97(1) | |
| Leu | | | 0.97(1) |
| Tyr | | | 1.44(2) |
| Phe | 0.77(1) | | |
| Lys | 0.81(1) | 2.10(2) | 1.93(2) |
| His | | | |
| Arg | 0.64(1) | | |
| Trp | - | +(1) | - |
| Yield(%) | 9.0 | 10.4 | 10.5 |
| Residue | 41-50 | 53-63 | 70-78 |

| | AC-1 | AC-2 | AC-3 | AC-4 | AC-5 | AC-6 |
|---|---|---|---|---|---|---|
| Asp | | 2.00(2) | 0.49 | 1.07(1) | 2.05(2) | |
| Thr | 0.96(1) | 1.09(1) | 0.97(1) | | | 1.05(1) |
| Ser | | 0.59 | | | 0.32 | 0.96(1) |
| Glu | 1.26(1) | 0.47 | 0.29 | 2.42(2) | 1.47(1) | |
| Pro | | | | 1.07(1) | 1.00(1) | |
| Gly | 3.25(3) | 0.65 | 0.84(1) | | 1.84(2) | |
| Ala | 1.98(2) | 1.58(2) | 0.87(1) | | 1.62(2) | |
| Val | | 0.26 | | | | |
| Met | | | | | | |
| Ile | | | 1.13(1) | 0.52(1) | 0.91(1) | |
| Leu | | | | 0.98(1) | 1.85(2) | 0.99(1) |
| Tyr | | 0.61(1) | 0.24 | 1.96(2) | 0.78(1) | |
| Phe | 0.95(1) | | | 0.63(1) | | |
| Lys | 1.11(1) | 0.98(1) | 1.29(1) | 2.24(2) | 2.30(2) | 2.23(2) |
| His | | 0.30 | | | | |
| Arg | 0.88(1) | | | | 0.70(1) | |
| Trp | - | - | +(1) | - | - | - |
| Yield(%) | 60.4 | 7.2 | 10.8 | 42.4 | 12.2 | 35.9 |
| Residue | 41-50 | 51-57 | 58-63 | 70-78 | 87-101 | 102-107 |

Table III-II. Amino acid compositions of chymotryptic peptides of larval- and adult-type cytochromes c. Amino acid analyses were performed on 24-h hydrolysates. Values in parentheses were taken from the sequence. Tryptophan was detected by its fluorescence.

Table III-III. The peptides LS-2, LS-3, LS-7, AS-2, AS-4 and AS-7 were contaminated with minor peptides. Because of a small amount of the peptides, they were used for sequence studies as a mixture without further purification. Although two PTH-amino acids were detected at all steps in the degradation of these peptides, the PTH-amino acids released from the minor peptides were easily detected by the area of the elution curves in HPLC. Edman degradation of each peptide fraction is summarized in Table III-IV.

Sequence studies of the larval- and adult-type cytochromes c of the housefly are summarized in Figs. III-3 A and B. The total number of the amino acid residues of each isocytochrome c was 107, and the molecular weights were calculated to be 12,155 and 12,253, including heme c for the larval and adult proteins, respectively. Six amino acid differences were obtained between the two proteins; they were the residues 13, 58, 93, 96 and 107.

## 3) DISCUSSION

In the previous study [13], we have inferred that the amino acid sequences of larval- and adult-type cytochromes c differs from each other in five or six residues on the basis of the amino acid compositions of the tryptic peptides. Although the sequence of the residues 64-65 was presumed to be Gln-Asp at that time, the sequence was determined to be Asn-Glu by the present study. The amino acid difference of the C-terminus causes difference in electrophoretic mobility between the two proteins (Fig. II-2);

| | LS-1 | LS-2 | LS-3 | LS-4 | LS-5 | LS-6 | LS-7 |
|---|---|---|---|---|---|---|---|
| Cmc | | | | | | | |
| Asp | 1.51(1) | 3.16(3) | 2.27(2) | 0.99(1) | 3.00(3) | 2.22(2) | |
| Thr | 1.18(1) | 1.94(2) | 0.62(1) | 0.93(1) | 1.91(2) | 1.76(2) | 1.07(1) |
| Ser | | 0.94(1) | 0.96(1) | | 0.92(1) | 0.81(1) | 0.97(1) |
| Glu | 0.59 | 1.86(2) | 0.99(1) | 1.09(1) | 1.03(1) | 0.32 | |
| Pro | 1.22(1) | | 0.48 | | 2.62(3) | 1.26(1) | |
| Gly | 5.31(6) | 2.33(2) | 0.83(1) | | 3.13(3) | 2.17(2) | |
| Ala | 1.56(1) | 3.98(4) | 0.70(1) | | 4.96(5) | 4.66(5) | 3.25(3) |
| Val | 1.05(1) | | | | | | |
| Met | | | | | 0.84(1) | 0.62(1) | |
| Ile | 0.35 | 1.27(1) | 1.23(1) | | 2.85(3) | 2.04(2) | 1.01(1) |
| Leu | 1.86(2) | | | 1.00(1) | 3.77(4) | 3.02(3) | 1.86(2) |
| Tyr | | 1.06(1) | | | 2.47(3) | 1.13(1) | 0.66(1) |
| Phe | 1.09(1) | 1.12(1) | | 0.92(1) | 1.02(1) | 0.90(1) | |
| Lys | 3.35(3) | 1.60(2) | 2.24(2) | | 5.30(6) | 3.93(4) | 0.65(1) |
| His | 1.76(2) | 0.55 | | | | | |
| Arg | 1.04(1) | 0.36 | | | | | |
| Trp | | (1) | (1) | | | | |
| Yield(%) | 11.5 | 6.7 | 7.8 | 40.8 | 15.4 | 8.6 | 9.9 |
| Residue | 26-45 | 46-65 | 55-65 | 66-70 | 71-107 | 82-107 | 98-107 |

| | AS-1 | AS-2* | AS-3 | AS-4* | AS-5 | AS-6 | AS-7* | AS-8 |
|---|---|---|---|---|---|---|---|---|
| Cmc | | | | | | | | |
| Asp | 1.41(1) | 1.58(2) | 1.04(1) | 1.70(1) | 1.11(1) | 1.18(1) | 1.50(1) | 0.42 |
| Thr | 0.32 | 0.92(1) | 0.97(1) | 1.02(1) | 0.87(1) | | 1.18(1) | 1.18(1) |
| Ser | | 0.40 | | | | | 0.81(1) | 1.01(1) |
| Glu | 0.35 | 1.20(1) | 1.06(1) | 1.52(1) | 0.95(1) | 1.14(1) | | 0.38 |
| Pro | 1.33(1) | | | 1.87(2) | 1.28(1) | 0.96(1) | | 0.50 |
| Gly | 5.24(5) | 1.32(1) | | 1.80(2) | 1.52(1) | 0.36 | 1.41(1) | 0.54 |
| Ala | 1.75(1) | 1.95(2) | | 1.13(1) | 1.35(1) | 0.22 | 2.52(2) | 2.35(2) |
| Val | 1.13(1) | | | | | | | |
| Met | | | | 0.38(1) | (1) | | | |
| Ile | 0.31 | 0.96(1) | | 1.71(2) | 0.81(1) | | 0.75(1) | 1.16(1) |
| Leu | 2.19(2) | | 0.92(1) | 1.14(1) | 1.18(1) | 0.89(1) | 1.35(2) | 2.10(2) |
| Tyr | | 0.44 | 1.14(2) | | | | 0.58(1) | 0.94(1) |
| Phe | 1.15(1) | | 0.99(1) | 0.50(1) | 0.77(1) | | | |
| Lys | 2.49(2) | 1.65(2) | | 2.87(3) | 2.63(3) | 1.58(2) | 1.48(2) | 2.46(2) |
| His | 2.47(2) | | | | | | | |
| Arg | 0.95(1) | | | | | | | |
| Trp | | (1) | | | | | | |
| Yield(%) | 13.4 | 11.5 | 31.7 | 10.6 | 4.3 | 4.7 | 6.5 | 6.7 |
| Residue | 26-42 | 55-65 | 66-70 | 71-88 | 82-94 | 89-94 | 95-107 | 98-107 |

Table III-III. Amino acid compositions of staphylococcal protease peptides of larval- and adult-type cytochromes c. Amino acid analyses were performed on 24-h hydrolysates. Tryptophan was not determined. Values in parentheses were taken from the sequence.
* Values were calculated by subtracting the compositions of their contaminant peptides.

| STEP | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| LS-2 | Gln / Tyr | Ala / Leu | Ala / Glu | Gly / Asn | Phe | Ala | Tyr | Thr | Asp | Ala |
| LS-3 | Ala | Asn / Gly | Lys / Gly | Ser / Lys | Lys / (His) | Gly / Lys | Ile / Val | | | |
| LS-7 | Leu / Ala | Ile / Gly | Ala / Gly | Tyr / Lys | Leu / (His) | Lys | (Ser) / Val | Ala / gly | | |
| AS-2 | Ala | Asn / Gly | Lys / Gly | Ala / LYS | Lys / (His) | Gly / Lys | Ile / Val | Thr / Gly | | |
| AS-4 | Tyr / Lys | Leu / Thr | Glu / Gly | Asn / Gln | Pro / Ala | Lys / Ala | Lys / Gly | Tyr / Phe | Ile / Ala | |
| AS-7 | Arg / Tyr | Gly / Leu | Asp / Glu | Leu / Asn | Ile / Pro | Ala / Lys | Tyr / Lys | | | |

Table III-IV. PTH-amino acids detected by Edman degradations of the peptide fractions from staphylococcal protease digests.

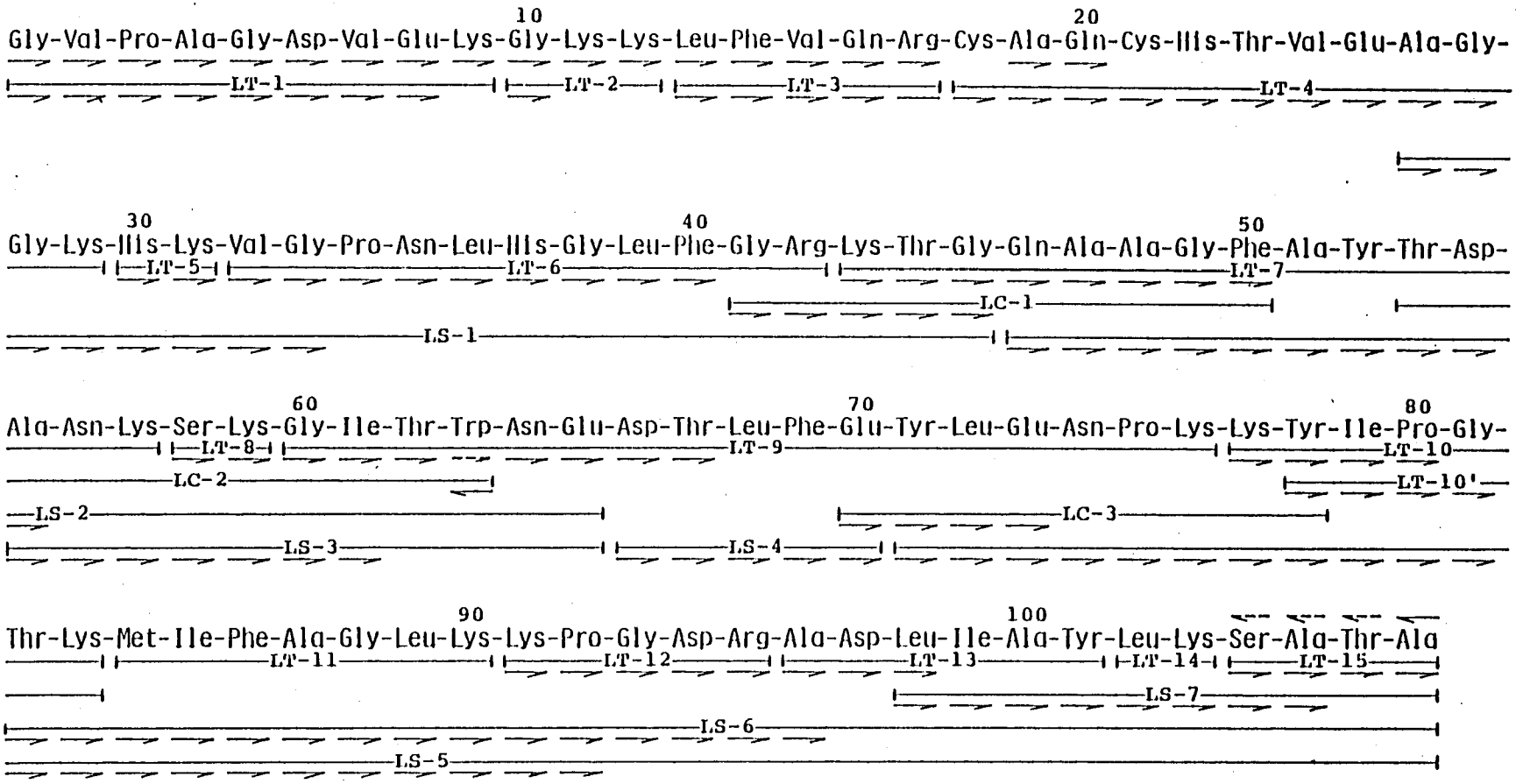


Fig. III-3(a). Summary of sequence studies of larval-type cytochrome c of the housefly.

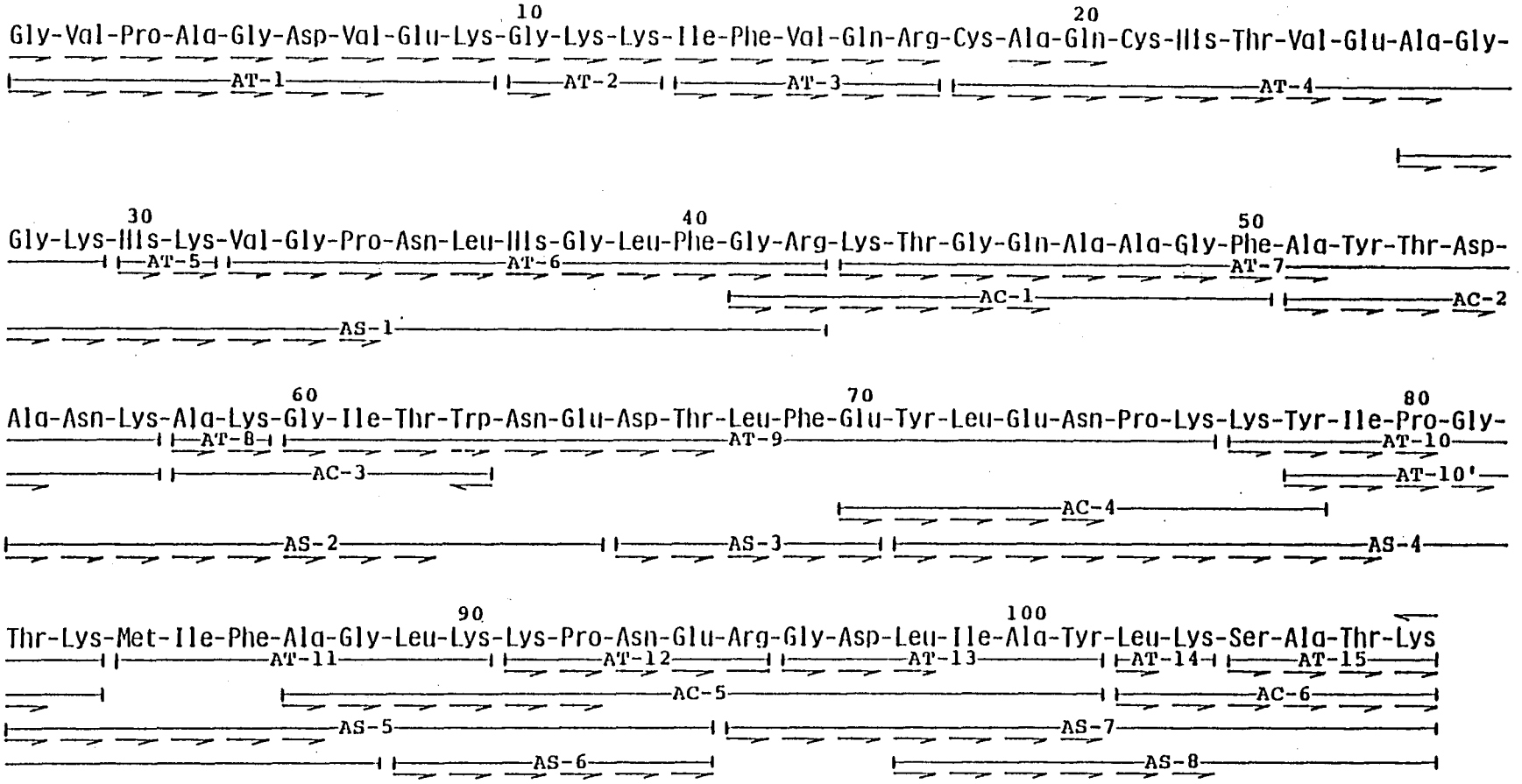


Fig. III-3(b). Summary of sequence studies of adult-type cytochrome c of the housefly. -T-, -C- and -S- refer to tryptic peptides, chymotryptic peptides and staphylococcal protease peptides, respectively. The amino acid sequence was determined by manual Edman degradation (——>) and carboxypeptidase digestion (<——). A line (——) shows the direct identification of C-terminal amino acid using the amino acid analyzer after completion of the degradation steps. Cys was determined as Cm-cysteine.

larval-type cytochrome c with alanine as the C-terminal residue is more acidic than adult-type cytochrome c with lysine as the terminal residue. In section IV.2, it will be discussed for evolutional relationships of these isocytochromes c in detail.

## 2. SEQUENCE STUDIES OF CYTOCHROMES C OF FRUIT FLY AND FLESH FLY

### 1)MATERIALS AND METHODS

#### Materials

The enzymes and chemicals used for the sequence determination in the present experiment were described in the privious papers [46,47].

#### Preparation of Fruit Fly and Flesh Fly Cytochromes c

Cytochrome c was purified from the fruit fly and the flesh fly as described in section II.2. S-Carboxymethyl(Cm)-cytochrome c was prepared by the method of Crestfield et al. [48] after removal of heme according to the method of Fontana et al. [49].

#### Amino Acid Sequence Studies

The amino acid compositions of Cm-cytochrome c and peptides were determined with an amino acid analyzer (IRICA model A-3300) after the sample was hydrolyzed with 6 N HCl for 24 h at 110°C in evacuated sealed tubes. Both manual and automated Edman degradation were used to determine the N-terminal sequences of Cm-cytochrome c and peptides. Automated Edman degradation was performed with an LKB 4030 solid-phase sequencer after the peptides had been coupled to aminopropyl-grass by p-phenylene-diisothiocyanate activation of the peptides [55,56]. PTH-amino acid were generally identified by HPLC [50], and in some cases by

TLC with the solvent V [51]. C-terminal sequences of cytochrome c were determined by carboxypeptidase digestion [53]; protein was digested by carboxypeptidase B for 30 min in 0.1 M Tris-HCl buffer (pH 8.0) at 40°C and the amino acids released were determined with amino acid analyzer.

Enzymatic Digestions and Separation of Peptides

One mg of cytochrome c was digested in 0.1 M $NH_4HCO_3$ with 20 μg of trypsin for 3.5 h at 40°C. The digests thus obtained were separated by HPLC in 0.1% trifluoroacetic acid with a linear gradient from 0 to 70% acetonitrile on an octadecylsilane column (IRICA RP-18, 4 x 250 mm, 0.8 ml/min) at 60°C according to the methods previously described [35]. Some peptides were further purified by high-voltage paper electrophoresis at pH 6.5 or 3.6 and by paper chromatography (butanol : pyridine : acetic acid : water = 15 : 10 : 3 : 12 in volume).

Nomenclature

T- refers to tryptic peptides of both fruit fly and flesh fly cytochromes c.

## 2) RESULTS

The Sequence Studies of Fruit Fly Cytochrome c

The amino acid composition of Cm-cytochrome c of the fruit fly is shown in Table III-V. The compositions obtained were in good agreement with that deduced from the complete sequence as

| | Fruit fly | | Flesh fly | |
|---|---|---|---|---|
| | Cm-cyt c | Sequence | Cm-cyt c | Sequence |
| Cmc | 2.05 | 2 | 2.02 | 2 |
| Asp | 9.98 | 9 | 9.58 | 9 |
| Thr | 7.58 | 7 | 7.25 | 7 |
| Ser | 1.28 | 1 | 1.07 | 1 |
| Glu | 10.3 | 9 | 10.0 | 9 |
| Pro | 5.27 | 5 | 5.67 | 6 |
| Gly | 14.8 | 14 | 14.5 | 14 |
| Ala | 11.3 | 11 | 10.3 | 10 |
| Val | 5.37 | 5 | 5.56 | 5 |
| Met | 1.32 | 1 | 1.23 | 1 |
| Ile | 5.40 | 5 | 5.47 | 5 |
| Leu | 8.11 | 8 | 6.84 | 7 |
| Tyr | 3.76 | 4 | 3.61 | 4 |
| Phe | 4.08 | 4 | 4.74 | 5 |
| Lys | 14.3 | 15 | 14.3 | 15 |
| His | 2.74 | 3 | 2.69 | 3 |
| Arg | 2.96 | 3 | 3.08 | 3 |
| Trp | n.d.* | 1 | n.d.* | 1 |
| Total | | 107 | | 107 |

Table III-V Amino acid compositions of Cm-cytochromes c of the fruit fly and the flesh fly. The amino acid analyses were performed on 24-h hydrolysates. The composition based on the sequence is also included. * Not determined.

described below. The solid-phase Edman degradation give the N-terminal sequence of Cm-cytochrome c as X-Val-Pro-Ala-Gly-Asp-Val-Glu-X-Gly-X-X-Leu-Phe-Val-Gln-Arg-Cmc-Ala-Gln-Cmc-. The residues 1-2 were determined to be Gly-Val- by manual Edman degradation. Carboxypeptidase B released lysine (0.38 mol / mol protein) after 30 min incubation, suggesting the C-terminal residue to be Lys.

Tryptic digest of fruit fly cytochrome c was separated by HPLC (Fig. III-4). Peptides T-1, T-2, T-3, T-7', T-8, T-10, T-10', T-12, T-13" and T-14 were further purified by paper electrophoresis at pH 6.5 and peptide T-5 was purified first by paper electrophoresis at pH 3.6, and then by paper chromatography. The amino acid compositions of the tryptic peptides are shown in Table III-VI.

Peptides T-5, T-6, T-8, T-12 and T-14 were completely sequenced by manual Edman degradation. As the amino acid compositions of peptides T-1, T-2 and T-3 agreed with that deduced from N-terminal sequence, their sequence studies were not performed. PTH-tryptophan was not identified at the 4th step of manual Edman degradation of peptide T-9, but it was concluded that this residue was tryptophan because the peptide showed tryptophan fluorescence and all cytochromes c so far sequenced have tryptophan at this position. Although no attempt to overlap these tryptic peptides was made, the alignment from T-1 to T-14 in this order from the N-terminus to the C-terminus was obvious from the sequence homology to other insect cytochromes c.

The sequence studies of fruit fly cytochrome c described above are summarized in Fig. III-5. The total number of the

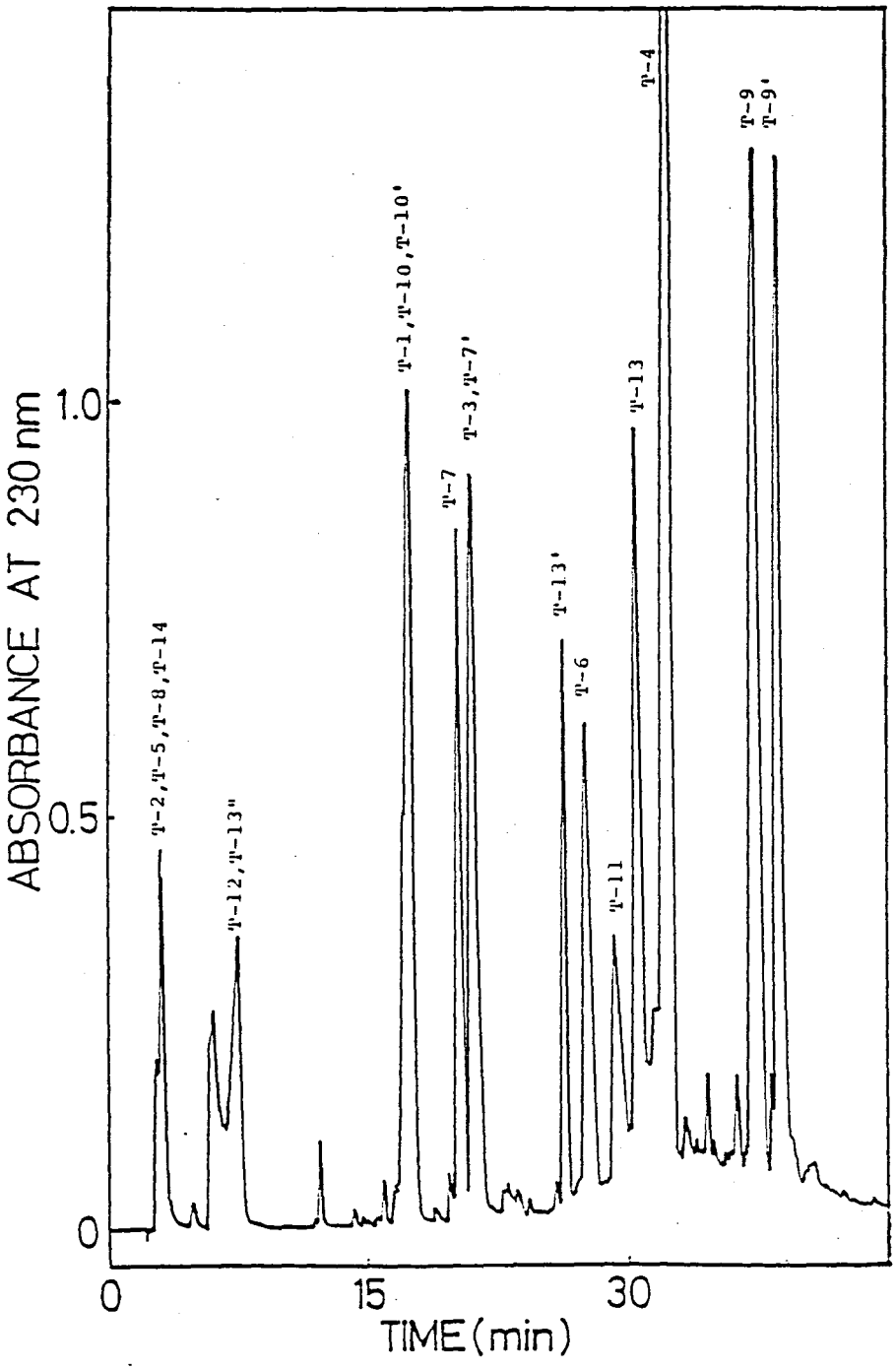


Fig. III-4. Separation of tryptic digests of fruit fly cytochrome c. The digests were chromatographed by HPLC under the conditions described in MATERIALS AND METHODS.

| Amino acid | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | T-7' | T-8 |
|---|---|---|---|---|---|---|---|---|---|
| Cys | | | | (2) | | | | | |
| Asp | 1.08(1) | 0.16 | | 0.12 | 0.18 | 1.04(1) | 2.01(2) | 2.23(2) | |
| Thr | | | | 1.00(1) | | | 2.06(2) | 1.95(2) | |
| Ser | | 0.57 | | 0.10 | 0.25 | | | | |
| Glu | 1.14(1) | 0.39 | 1.29(1) | 2.11(2) | 0.22 | | 1.07(1) | 1.25(1) | |
| Pro | 0.93(1) | | | | | 0.91(1) | | | |
| Gly | 1.70(2) | 1.14(1) | 0.16 | 2.15(2) | 0.44 | 3.09(3) | 2.12(2) | 1.81(2) | |
| Ala | 1.01(1) | 0.18 | | 1.89(2) | | | 3.78(4) | 4.35(4) | 0.99(1) |
| Val | 2.15(2) | | 1.21(1) | 1.06(1) | 0.29 | 1.03(1) | | | |
| Met | | | | | | | | | |
| Ile | | | | | | 1.01(1) | | | |
| Leu | | | 0.56(1) | | 0.14 | 1.90(2) | | | |
| Tyr | | | | | | | 0.93(1) | 0.79(1) | |
| Phe | | | 0.99(1) | | | | 0.95(1) | 0.97(1) | |
| Lys | 0.92(1) | 1.72(2) | | 0.99(1) | 1.04(1) | | 1.93(2) | 0.89(1) | 1.01(1) |
| His | | | | 0.93(1) | 0.96(1) | 0.95(1) | | | |
| Arg | | | 1.16(1) | | | 1.03(1) | | | |
| Trp | | | | | | | | | |
| Total | 9 | 3 | 5 | 12 | 2 | 11 | 15 | 14 | 2 |
| Yield(%) | 60.9 | 26.0 | 34.9 | 46.9 | 10.3 | 73.1 | 38.6 | 15.4 | 45.7 |

Table III-VI. Amino acid compositions of tryptic peptides of fruit fly cytochrome c. Amino acid analyses were performed on 24-h hydrolysates. Tryptophan was detected by its fluorescence. Values in parentheses were taken from the sequence.

| Amino acid | T-9 | T-9' | T-10 | T-11 | T-12 | T-13 | T-13' | T-13" | T-14 |
|---|---|---|---|---|---|---|---|---|---|
| Cys | | | | | | | | | |
| Asp | 2.96(3) | 2.91(3) | | | 1.10(1) | 0.97(1) | 1.01(1) | 0.11 | |
| Thr | 1.93(2) | 1.87(2) | 1.06(1) | | | 0.11 | | | 1.04(1) |
| Ser | 0.12 | 0.14 | | | | | | 0.21 | 0.69(1) |
| Glu | 3.13(3) | 3.11(3) | | | 1.20(1) | 0.20 | | 0.21 | |
| Pro | 0.94(1) | 0.89(1) | 0.97(1) | | 0.96(1) | | | | |
| Gly | 1.16(1) | 1.17(1) | 1.12(1) | 1.13(1) | 0.15 | 1.18(1) | 1.15(1) | 0.36 | |
| Ala | 0.17 | 0.19 | | 1.00(1) | | 1.03(1) | 0.91(1) | | 1.09(1) |
| Val | | | | | | | | | |
| Met | | | | 0.98(1) | | | | | |
| Ile | 0.95(1) | 0.93(1) | 0.96(1) | 1.02(1) | | 0.89(1) | 0.93(1) | | |
| Leu | 1.80(2) | 1.76(2) | | 0.95(1) | | 1.69(2) | 0.92(1) | 0.80(1) | |
| Tyr | 0.86(1) | 0.90(1) | 0.46(1) | | | 0.73(1) | 0.75(1) | | |
| Phe | 0.96(1) | 0.95(1) | | 0.91(1) | | | | | |
| Lys | 1.89(2) | 1.00(1) | 0.88(1) | 1.01(1) | 0.64(1) | 0.98(1) | 0.10 | 1.20(1) | 0.91(1) |
| His | | | | | | | | | |
| Arg | | | | | 1.06(1) | | | | |
| Trp | + (1) | + (1) | | | | | | | |
| Total | 18 | 17 | 6 | 7 | 5 | 8 | 6 | 2 | 4 |
| Yield | 43.6 | 32.6 | 36.4 | 85.4 | 51.3 | 53.2 | 24.9 | 8.2 | 66.2 |

Table III-VI. Amino acid compositions of tryptic peptides of fruit fly cytochrome c. (continued).

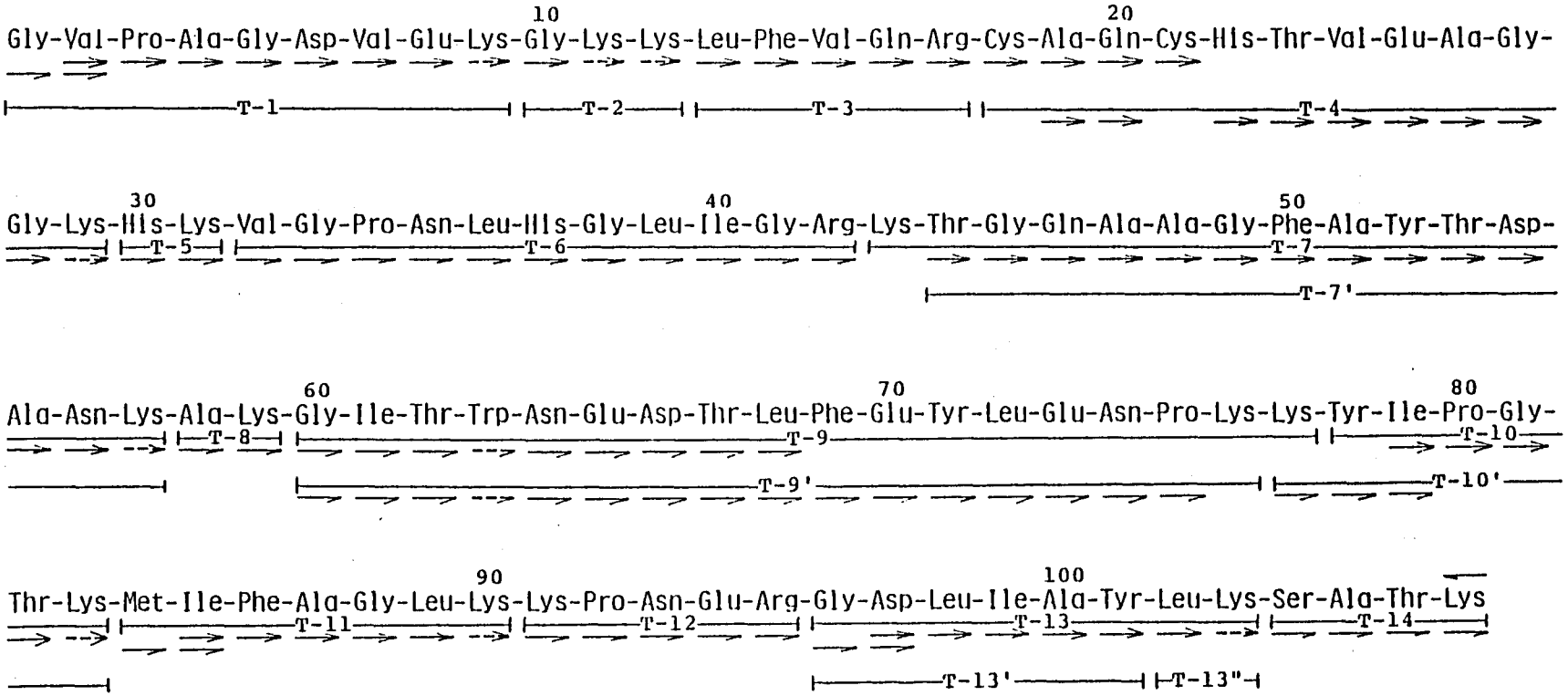


Fig. III-5. Summary of sequence studies of fruit fly cytochrome c. T- refers to tryptic peptide. The amino acid sequence was determined by manual Edman degradation (⟶), solid-phase Edman degradation (⟶) and carboxypeptidase digestion (⟵). Cys was determined as Cm-cysteine.

amino acid residues was 107, giving a molecular weight of 12,220 including heme c.

The Sequence Studies of Flesh Fly Cytochrome c

The amino acid composition of Cm-cytochrome c of the flesh fly is shown in Table III-V. The compositions were in good agreement with that deduced from the complete sequence as described below. By the solid-phase Edman degradation, the N-terminal sequence of Cm-cytochrome c was determined to be X-Val-Pro-Ala-Gly-Asp-Val-Glu-X-Gly-X-X-Ile-Phe-Val-Gln-Arg-Cmc-Ala-Gln-Cmc-X-Thr-Val-Glu-Ala-Gly-Gly-. The residues 1-2 was determined to be Gly-Val- by manual Edman degradation. Carboxypeptidase B released lysine (0.67 mol / mol protein) after 30 min incubation, suggesting the C-terminal residue to be Lys.

Tryptic digest of flesh fly cytochrome c was separated by HPLC (Fig. III-6). Peptides T-2, T-8, T-12 and T-14 were further purified by paper electrophoresis at pH 6.5, and the peptide T-6 and T-11 were similarly purified at pH 3.6, and peptide T-5 was purified first by paper electrophoresis at pH 3.6, and then by paper chromatography. The amino acid compositions of the tryptic peptides are shown in Table III-VII.

Peptides T-5, T-6, T-8 and T-12 were completely sequenced by manual Edman degradation. As the amino acid compositions of peptides T-1, T-2 and T-3 agreed with those deduced from the N-terminal sequence, their sequence studies were not performed. Although no attempt to overlap these tryptic peptides was made, the alignment from T-1 to T-14 in this order from the N-terminus to the C-terminus was obvious from the sequence homology to other

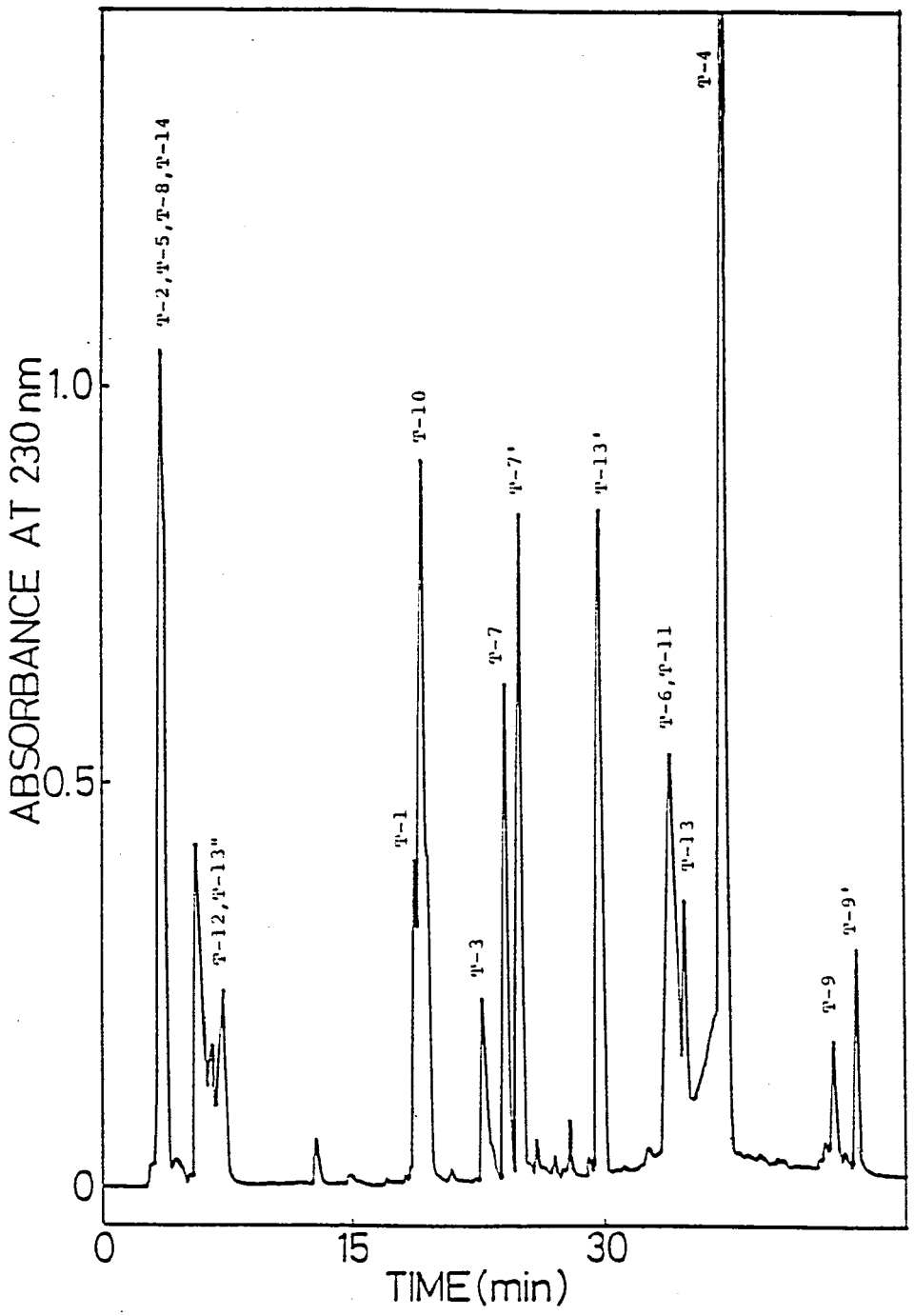


Fig. III-6. Separation of tryptic digests of flesh fly cytochrome c. The digests were fractionated by HPLC as described in MATERIALS AND METHODS.

| Amino acid | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | T-7' | T-8 |
|---|---|---|---|---|---|---|---|---|---|
| Cys | | | | (2) | | | | | |
| Asp | 1.01(1) | | | | | 1.27(1) | 1.97(2) | 2.00(2) | |
| Thr | | | | 1.08(1) | | 0.12 | 1.96(2) | 1.95(2) | |
| Ser | | | | | | 0.42 | | | |
| Glu | 1.09(1) | | 1.08(1) | 2.14(2) | | 0.59 | 1.13(1) | 1.11(1) | |
| Pro | 0.91(1) | | | | | 1.08(1) | 0.90(1) | 0.93(1) | |
| Gly | 2.00(2) | 0.83(1) | | 2.13(2) | 0.18 | 3.42(3) | 2.09(2) | 2.10(2) | |
| Ala | 0.92(1) | | | 1.92(2) | | 0.26 | 2.78(3) | 2.81(3) | 1.02(1) |
| Val | 1.98(2) | | 0.99(1) | 1.09(1) | | 1.10(1) | | | |
| Met | | | | | | | | | |
| Ile | | | 0.93(1) | | | | | | |
| Leu | | | | | | 2.01(2) | | | |
| Tyr | | | | | | | 0.86(1) | 0.71(1) | |
| Phe | | | 0.91(1) | | | 0.97(1) | 0.97(1) | 0.94(1) | |
| Lys | 0.97(1) | 2.34(2) | | 0.97(1) | 1.18(1) | | 1.88(2) | 0.97(1) | 0.98(1) |
| His | | | | 0.93(1) | 0.82(1) | 0.93(1) | | | |
| Arg | | | 1.01(1) | | | 1.10(1) | | | |
| Trp | | | | | | | | | |
| Total | 9 | 3 | 5 | 12 | 2 | 11 | 15 | 14 | 2 |
| Yield(%) | 51.4 | 13.4 | 70.9 | 36.0 | 17.8 | 11.7 | 24.0 | 52.0 | 39.0 |

Table III-VII. Amino acid compositions of tryptic peptides of flesh fly cytochrome c. Amino acid analyses were performed on 24-h hydrolysates. Tryptophan was detected by its fluorescence. Values in parentheses were taken from the sequence.

| Amino acid | T-9 | T-9' | T-10 | T-11 | T-12 | T-13 | T-13' | T-13" | T-14 |
|---|---|---|---|---|---|---|---|---|---|
| Cys | | | | | | | | | |
| Asp | 2.64(3) | 3.06(3) | 0.30 | 0.51 | 1.12(1) | 1.09(1) | 1.01(1) | | |
| Thr | 1.70(2) | 1.79(2) | 0.88(1) | | | 0.16 | | | 1.01(1) |
| Ser | 0.39 | | | 0.28 | 0.12 | 0.10 | | | 0.65(1) |
| Glu | 3.92(3) | 3.03(3) | 0.32 | 0.28 | 1.22(1) | 0.38 | | | |
| Pro | 1.24(1) | 0.99(1) | 1.10(1) | 0.20 | 0.93(1) | | | | |
| Gly | 1.56(1) | 0.89(1) | 1.45(1) | 1.78(1) | 0.27 | 1.76(1) | 1.09(1) | 0.18 | |
| Ala | 0.53 | | 0.26 | 1.21(1) | | 0.99(1) | 0.96(1) | | 1.14(1) |
| Val | 1.01 | | 0.58 | 0.15 | | 0.61 | | | |
| Met | | | | 0.60(1) | | | | | |
| Ile | 0.64(1) | 0.82(1) | 0.81(1) | 1.05(1) | | 0.69(1) | 0.95(1) | | |
| Leu | 1.67(2) | 1.95(2) | | 1.31(1) | | 1.89(2) | 0.94(1) | 0.80(1) | |
| Tyr | 0.76(1) | 1.27(1) | 0.74(1) | | | 0.42(1) | 0.86(1) | | |
| Phe | 0.69(1) | 1.00(1) | | 1.07(1) | | 0.11 | | | |
| Lys | 2.06(2) | 1.10(1) | 1.27(1) | 1.00(1) | 0.61(1) | 1.10(1) | | 1.20(1) | 0.86(1) |
| His | 0.41 | | | 0.16 | | 0.41 | | | |
| Arg | | | | 0.17 | 1.05(1) | | | | |
| Trp | + (1) | + (1) | | | | | | | |
| Total | 18 | 17 | 6 | 7 | 5 | 8 | 6 | 2 | 4 |
| Yield(%) | 8.0 | 10.0 | 76.7 | 15.2 | 28.1 | 12.0 | 28.4 | 14.7 | 33.2 |

Table III-VII. Amino acid compositions of tryptic peptides of flesh fly cytochrome c. (continued).

insect cytochromes c.

The sequence studies of flesh fly cytochrome c described above are summarized in Fig. III-7. The total number of the amino acid residues was 107, giving a molecular weight of 12,280 including heme c.

## 3) DISCUSSION

The sequence studies of fruit fly and flesh fly cytochromes c are summarized in Fig. III-5 and III-7, respectively. Three amino acid differences were observed between the two proteins; they were the residues 13, 40 and 48.

Cytochrome c of the fruit fly Drosophila melanogaster was already sequenced in 1968 by Nolan et al. [57]. When the sequence of the fruit fly cytochrome c determined in the present study was compared with the sequence determined by them, three amino acid differences were observed; the residues 54, 64 and 65. Three amino acid differences seem too large to be explained by the heterogeneity in the fruit fly. In fact, the sequence of fruit fly cytochrome c in this study agreed with that of mediterranean fruit fly cytochrome c [58]. The author concluded that the sequence by Margolish and his colleagues might be mistaken. Cytochromes c of the flies already sequenced were summarized in Fig. III-8. Cytochromes c of the screw-worm fly and the blowfly had Asn-54, Gln-64 and Asp-65, just as that of the fruit fly sequenced by Margolish and his colleagues. The author thought that they might be also mistaken. In the case of the blowfly

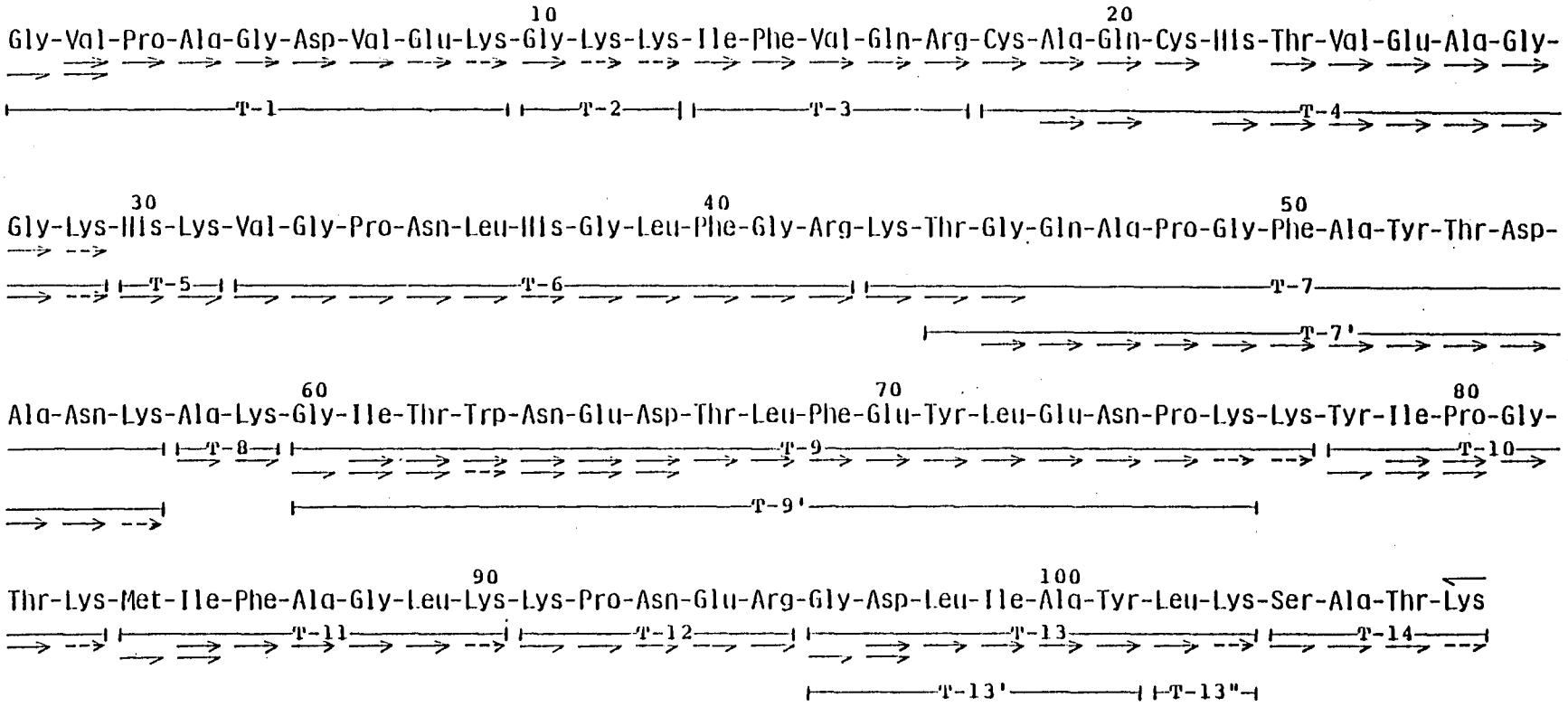


Fig. III-7. Summary of sequence studies of flesh fly cytochrome c. T- refers to tryptic peptide. The amino acid sequence was determined by manual Edman degradation (⟶), solid-phase Edman degradation (⟶) and carboxypeptidase digestion (⟵). Cys was determined as Cm-cysteine.

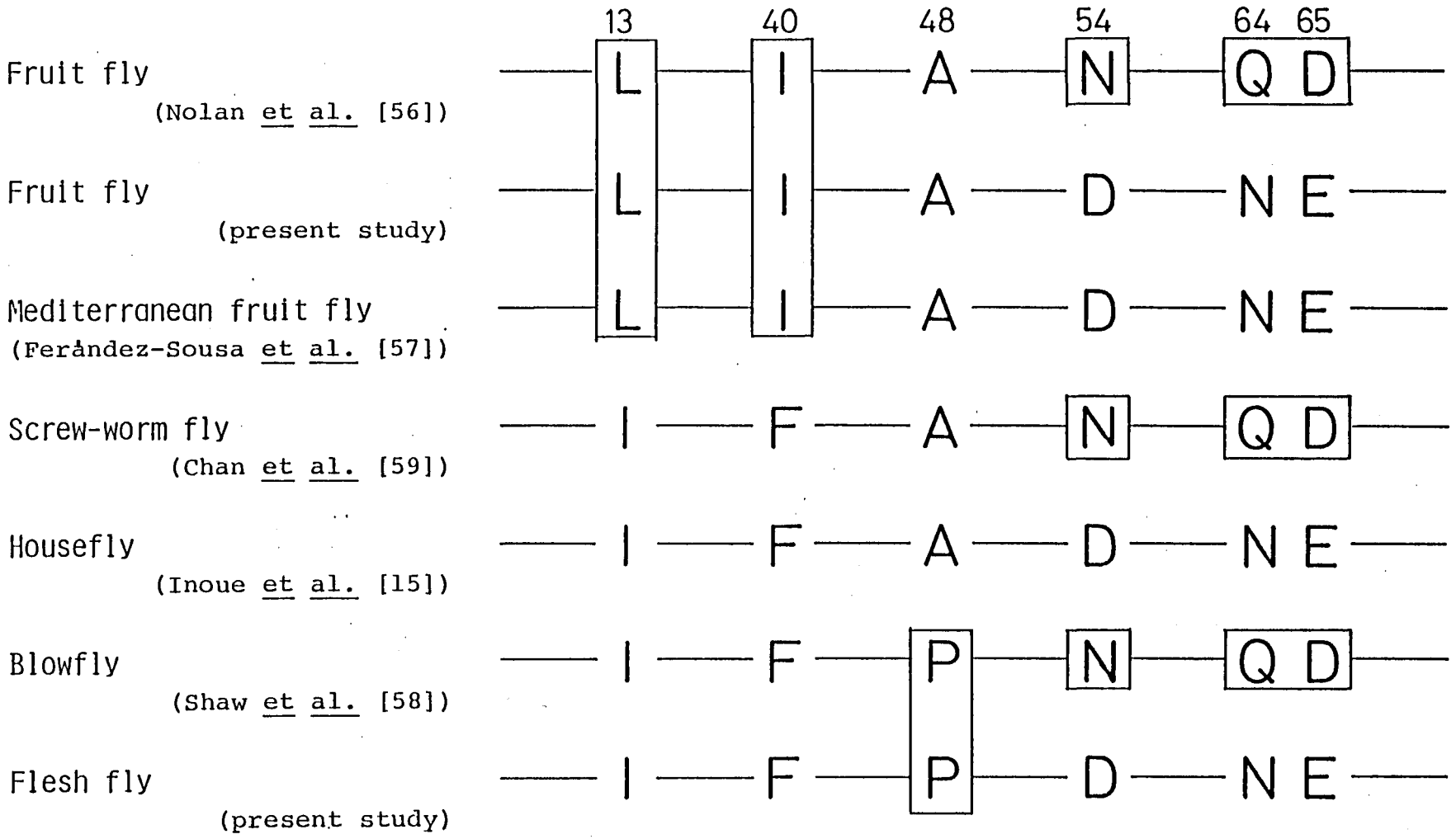


Fig. III-8. Sequence comparison of fly cytochromes c. The boxed residues indicate the amino acids which differ from those of adult-type cytochrome c of the housefly.

[58], its sequence has not been completely determined. It was presumed by the amino acid compositions of the tryptic peptides and sequence homology to fruit fly and screw-worm fly cytochromes c that had been already sequenced at that time. If the sequence homology used in determining the sequence of blowfly cytochrome c is based on the sequence of fruit fly cytochrome c determined in the present study, and on those of flesh fly, housefly and mediterranean fruit fly cytochromes c, its sequence will agree with that of flesh fly cytochrome c at the residues; Asp-54, Asn-64 and Glu-65. As the sequence of screw-worm fly cytochrome c detemined by Chan et al. [59] was unpublished results, the sequence might be based on the amino acid compositions of the tryptic peptide and the sequence homology to fruit fly cytochrome c, just as the case of that of the blowfly cytochrome. If the author's assumption is correct, the sequence of the screw-worm fly cytochrome c will have Asp at the residue 54, Asn at the residue 64 and Glu at the residue 65 and becomes identical with that of the housefly adult-type cytochrome c. Phylogenetic trees were constructed based on either the published sequences (Fig. III-9(A)) or those corrected in the present study (Fig. III-9(B)). Phylogenetic relationships based on the morphology are summarized in Fig. III-10. Phylogenetic relationships based on the morphology agreed well with the phylogenetic tree based on the corrected sequences, but not with that based on the published sequences. This will give the further suggestions that the sequences of the fruit fly and the screw-worm fly cytochromes c by Margoliash and his colleagues and the blowfly cytochrome c have been mistaken.

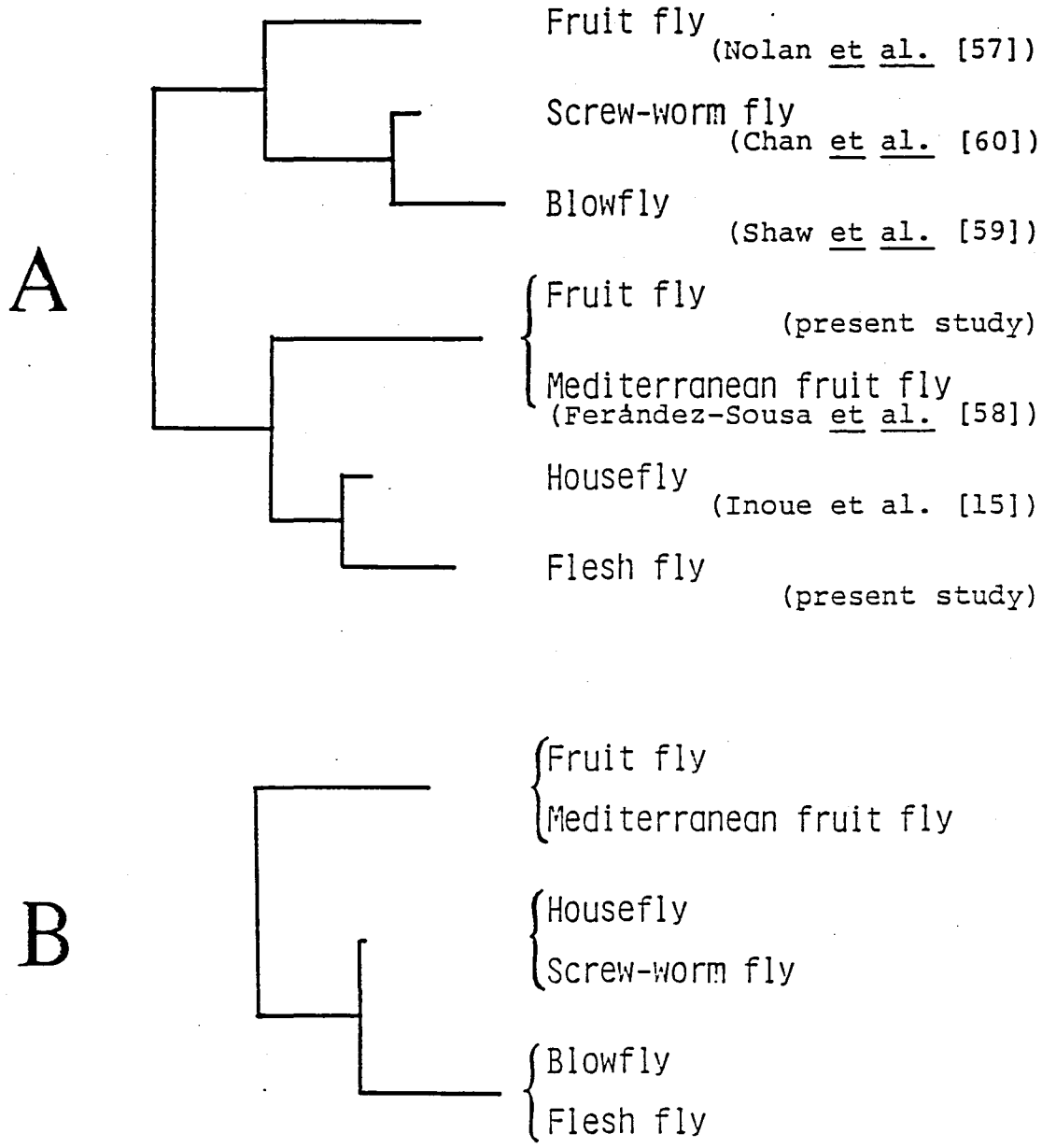


Fig. III-9. Phylogenetic trees of fly cytochromes c. Branch lengths were calculated using the amino acid difference matrices. (A) A phylogenetic tree based on the published sequences. (B) A phylogenetic tree based on the corrected sequences.

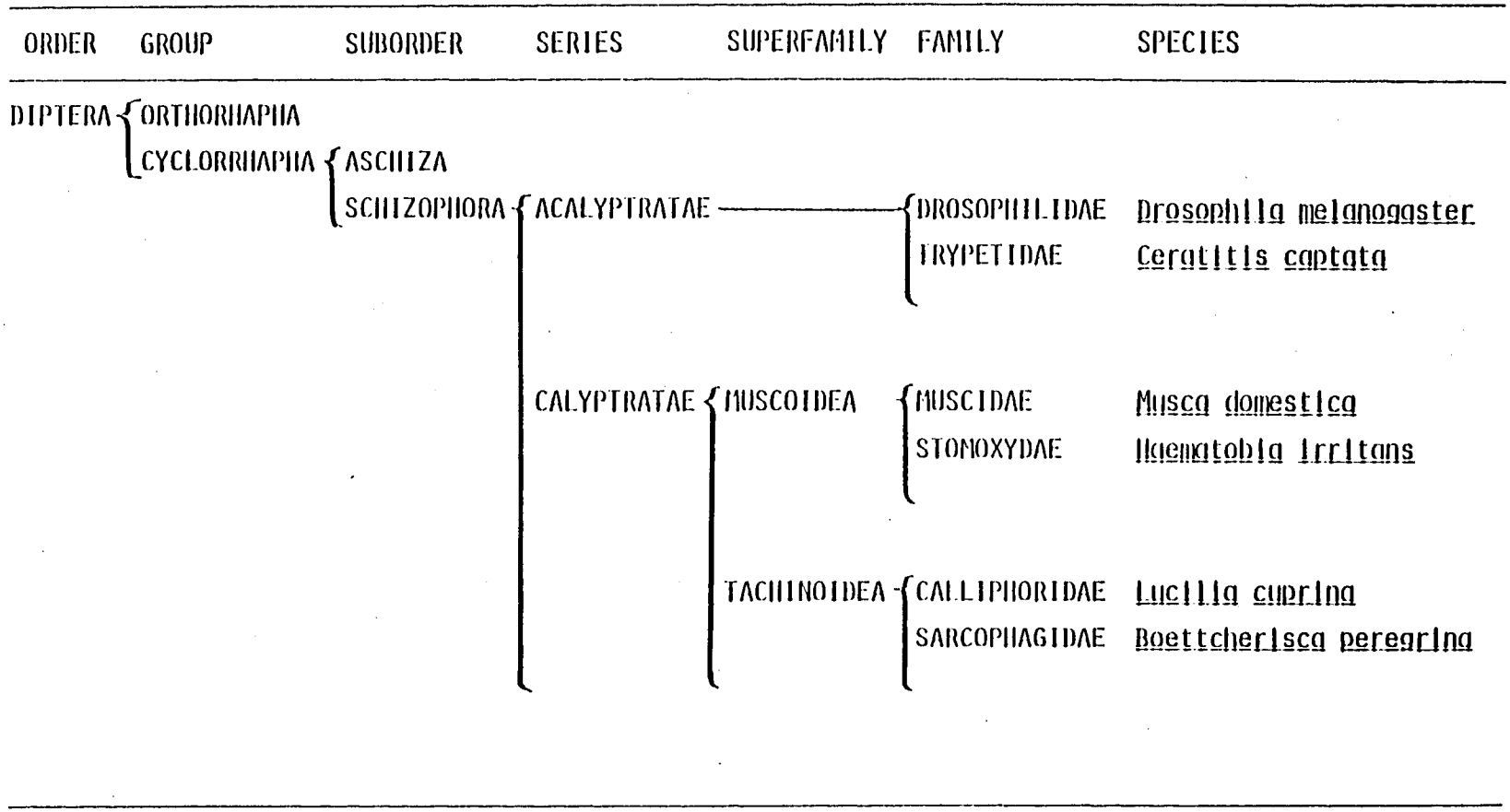


Fig. III-10. Phylogenetic relationships among flies based on the morphology.

## 3. SEQUENCE STUDIES OF HONEYBEE CYTOCHROME C

### 1)MATERIALS AND METHODS

#### Materials

The enzymes and chemicals used for the sequence determination in the present experiment were described in the privious papers [46,47].

#### Preparation of Honeybee Cytochrome c

Cytochrome c was purified by the methods previouly described [15]. About 200 g of frozen worker adults were homogenized in 0.5% $Al_2(SO_4)_3$ at pH 4.5. After 2 h, the resulting homogenate was centrifuged at 20,000 x g for 20 min. The supernatant obtained was neutralized by addition of $NH_4OH$, and then centrifuged to discard aluminium hydroxide precipitated. The supernatant thus obtained was passed through an Amberlite CG-50 column which had been equilibrated with 20 mM sodium phosphate buffer, pH 8.0. Cytochrome c adsorbed on the column was eluted with 20 mM sodium phosphate buffer, pH 8.0 containing 500 m NaCl. To the resulting eluate, was added ammonium sulfate powder to a final concentration of 90% saturation. After being centrifuged at 20,000 x g for 30 min, the supernatant obtained was passed through a small DEAE-cellulose-(DE-32, Whatman) column which had been equilibrated with 90% saturated ammonium sulphate. Cytochrome c adsorbed on the column was eluted with 20 mM

ammonium phosphate buffer, pH 6.8, and the eluate was dialyzed against the same buffer. The dialyzate obtained was charged on an Amberlite CG-50 column (1 cm x 30 cm) which had been equilibrated with the same buffer as used for the above dialysis. Cytochrome c adsorbed on the column was eluted with a linear gradient of ammonium phosphate from 20 mM to 300 mM. Cm-cytochrome c was prepared by the method of Crestfield et al. [48] after removal of heme according to the method of Fontana et al. [49].

Amino Acid Sequence Studies

The amino acid compositions of Cm-cytochrome c and peptides were determined with an amino acid analyzer (IRICA model A-3300) after the sample was hydrolyzed with 6 N HCl for 24 h at 110°C in evacuated sealed tubes. Manual Edman degradation method [52] was used to determine N-terminal sequence of Cm-cytochrome c and peptides. PTH-amino acid were generally identified by HPLC (IRICA) [50], and in some cases by TLC with the solvent V [51]. C-terminal sequences of cytochrome c were determined by carboxypeptidase B digestion in 0.1 M Tris-HCl buffer, pH 8.0, at 40°C [53] followed by detection of the amino acids released with amino acid analyzer.

Enzymatic Digestions and Separation of Peptides

One mg of cytochrome c was digested in 0.1 M $NH_4HCO_3$ with 20 $\mu g$ of trypsin for 3.5 h at 40°C and 1 mg of Cm-cytochrome c was digested with staphylococcal protease in the same buffer overnight at 40°C. The digests thus obtained were separated by

HPLC in 0.1% trifluoroacetic acid with a linear gradient from 0 to 70% acetonitrile on an octadecylsilane column (IRICA RP-18, 4 x 250 mm, 0.8 ml/min) at 60°C according to the methods previously described [35]. Some peptides were further purified by high-voltage paper electrophoresis at pH 6.5 or 3.6 and by paper chromatography (butanol : pyridine : acetic acid : water = 15 : 10 : 3 : 12 in volume). Heme peptide T-4 was separated from T-12′ on a small talc column using 50% pyridine as the elution solution.

Nomenclature

T- and S- refer to tryptic and staphylococcal protease peptides, respectively.

## 2) RESULTS

The N-terminal sequence of Cm-cytochrome c was determined by manual Edman degradation to be Gly-Ile-Pro-Ala-Pro-Glu-Lys-Gly-Lys-Lys-Ile-Phe-Val-Gln-Lys-Cmc-Ala-Gln-Cmc-His-Thr-Ile. Lysine was released from cytochrome c in 1 h-digestion (0.59 mol / mol protein) and 4 h-digestion (0.93) by carboxypeptidase B. Tryptic peptides were separated by HPLC (Fig. III-11). Peptides T-2, T-3, T-9, and T-13 were further purified by paper electrophoresis at pH 6.5, the peptide T-10 was similarly purified at pH 3.6, and the peptide T-5 was purified first by paper electrophoresis at pH 3.6, and then by paper chromatography. As heme peptide T-4 could not be separated from T-12′ by HPLC, it was further purified by

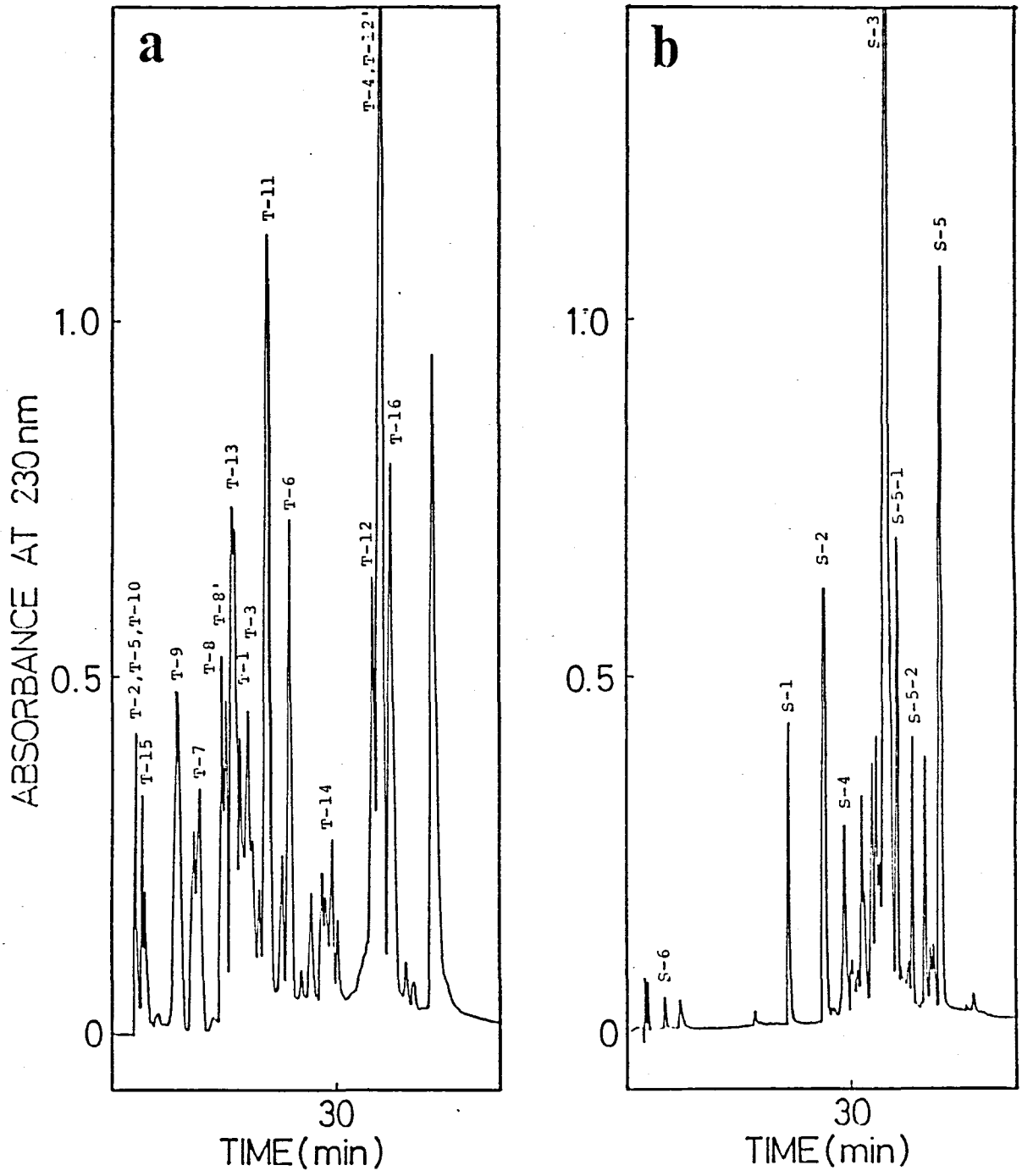


Fig. III-11. (a) Separation of tryptic digests of honeybee cytochrome c. The digests were chromatographed by HPLC under the conditions described in MATERIALS AND METHODS.

(b) Separation of staphylococcal protease digests of Cm-cytochrome c of honeybee. The digests were fractionated by HPLC as described in MATERIALS AND METHODS.

chromatography with a small talc column. The amino acid compositions of the tryptic peptides obtained are shown in Table III-VIII. The peptide T-10 was considered to be originated from two different parts of the cytochrome c molecule; positions 10-11 and 58-59. PTH-tryptophan was not identified by Edman degradation, but auther concluded that the residue at 63 was tryptophan because the peptide T-11 had showed tryptophan fluorescence and all cytochromes c so far known have tryptophan at this position.

Staphylococcal protease peptides were also purified by HPLC (Fig. III-11) and the peptides S-1 to S-6 were obtained. The amino acid compositions of these peptides are given in Table III-IX. The peptides S-3 and S-5 were subjected to the sequence studies.

The sequence studies of honeybee cytochrome c described above are summarized in Fig. III-12. The total number of the amino acid residues was 107, giving a molecular weight of 12,431 including heme c.

## 3) DISCUSSION

The amino acid composition and N-terminal residue of honeybee cytochrome c have been already determined by Carlson et al. [44]. Their results are consistent with those obtained in the present study. When all insect cytochromes c so far sequenced are compared (Fig. IV-2), many unique residues were found with honeybee cytochrome c; Ile-2, Lys-17, Ile-24, Ser-26,

| Amino acid | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | T-8 | T-8' |
|---|---|---|---|---|---|---|---|---|---|
| Cys | | | | (2) | | | | | |
| Asp | 1.03(1) | | | 0.19 | | 0.99(1) | 0.13 | | 0.10 |
| Thr | 0.12 | | | 1.01(1) | | | 0.10 | 1.08(1) | 0.99(1) |
| Ser | | | | 1.17(1) | | | 0.15 | 0.12 | |
| Glu | 1.15(1) | | 1.09(1) | 2.07(2) | | 0.12 | 0.19 | 1.15(1) | 1.01(1) |
| Pro | 1.93(2) | | | | | 1.06(1) | | 1.04(1) | 0.97(1) |
| Gly | 1.69(2) | 1.01(1) | 0.23 | 2.08(2) | 0.22 | 1.06(1) | 2.19(2) | 2.37(2) | 2.05(2) |
| Ala | 0.99(1) | | | 1.02(1) | | | | 1.15(1) | 0.96(1) |
| Val | | | 1.09(1) | 0.20 | | 0.96(1) | 1.15(1) | 0.17 | |
| Met | | | | | | | | | |
| Ile | 0.89(1) | | 0.89(1) | 0.95(1) | | | | | |
| Leu | 0.11 | | | 0.17 | | 0.95(1) | 0.11 | | |
| Tyr | 0.17 | | | | | 0.73(1) | 1.02(1) | 0.94(1) | 0.78(1) |
| Phe | | | 0.94(1) | | | | | | |
| Lys | 0.98(1) | 1.98(2) | 0.98(1) | 0.98(1) | 1.17(1) | 0.13 | | 0.91(1) | 0.15 |
| His | | | | 0.92(1) | 0.83(1) | | | | |
| Arg | | | | | | | 0.90(1) | | |
| Trp | | | | | | | | | |
| Total | 9 | 3 | 5 | 12 | 2 | 6 | 5 | 8 | 7 |
| Yield(%) | 47.2 | 20.9 | 33.6 | 13.3 | 18.7 | 33.1 | 20.2 | 17.2 | 20.0 |

Table III-VIII. Amino acid compositions of tryptic peptides of honey bee cytochrome c. Amino acid analyses were performed on 24-h hydrolysates. Tryptophan was detected by its fluorescence. Values in parentheses were taken from the sequence.

| Amino acid | T-9 | T-10 | T-11 | T-12 | T-12' | T-13 | T-14 | T-15 | T-16 |
|---|---|---|---|---|---|---|---|---|---|
| Cys | | | | | | | | | |
| Asp | 2.03(2) | | 0.99(1) | 1.16(1) | 1.00(1) | 0.10 | | | 1.05(1) |
| Thr | 1.01(1) | | 0.84(1) | 1.15(1) | 0.95(1) | 0.92(1) | | | 0.11 |
| Ser | 0.96(1) | | 0.34 | 0.38 | 0.13 | 0.16 | 0.10 | | 1.02(1) |
| Glu | | | 0.34 | 3.28(3) | 2.92(3) | 0.15 | 0.13 | 2.06(2) | 2.10(2) |
| Pro | | | | 1.00(1) | 0.94(1) | 0.89(1) | | 0.93(1) | |
| Gly | 0.11 | 0.96(1) | 1.16(1) | 0.78 | 0.18 | 1.09(1) | 1.14(1) | | 0.18 |
| Ala | 0.99(1) | | 0.13 | 0.52 | | 0.10 | 0.98(1) | | 2.87(3) |
| Val | | | 0.19 | 0.61 | | | 0.95(1) | | 0.16 |
| Met | | | | 0.16 | | | 0.81(1) | | |
| Ile | | | 0.83(1) | 0.22 | | 0.79(1) | | | 1.83(2) |
| Leu | | | 0.11 | 2.32(2) | 1.90(2) | | 0.98(1) | | 1.00(1) |
| Tyr | 0.68(1) | | | 0.80(1) | 0.59(1) | 0.71(1) | | | 0.85(1) |
| Phe | | | | 1.29(1) | 0.95(1) | | 0.92(1) | | |
| Lys | 0.99(1) | 1.04(1) | 0.85(1) | 1.98(2) | 1.13(1) | 0.91(1) | 0.98(1) | 1.01(1) | 0.94(1) |
| His | | | | | | | | | |
| Arg | | | | | | | | 0.96(1) | |
| Trp | | | + (1) | | | | | | |
| Total | 7 | 2 | 6 | 12 | 11 | 6 | 7 | 5 | 12 |
| Yield(%) | 36.1 | 47.9 | 44.7 | 5.2 | 18.4 | 22.4 | 25.0 | 29.7 | 34.9 |

Table III-VIII. Amino acid compositions of tryptic peptides of honey bee cytochrome c. (continued).

| Amino acid | S-1 | S-2 | S-3 | S-4 | S-5 | S-5-1 | S-5-2 | S-6 |
|---|---|---|---|---|---|---|---|---|
| Cmc | | 1.71(2) | | | | | | |
| Asp | 1.00(1) | | 4.00(4) | 0.11 | 2.03(2) | 1.30(1) | 1.10(1) | |
| Thr | | 0.93(1) | 2.92(3) | 0.99(1) | 1.01(1) | 1.10(1) | | |
| Ser | | | 2.07(2) | | | 0.25 | 0.13 | 0.93(1) |
| Glu | 1.10(1) | 3.46(3) | 2.46(2) | 1.15(1) | 4.63(4) | 3.03(3) | 1.18(1) | 1.10(1) |
| Pro | 1.91(2) | | 1.80(2) | | 2.89(3) | 2.66(3) | | |
| Gly | 1.96(2) | 0.80(1) | 8.77(9) | 0.29 | 2.05(2) | 2.19(2) | | |
| Ala | 0.92(1) | 0.94(1) | 1.90(2) | | 2.83(3) | 0.93(1) | 1.84(2) | 0.83(1) |
| Val | | 0.95(1) | 1.70(2) | 0.15 | 1.03(1) | 1.09(1) | | |
| Met | | | | | 0.49(1) | 0.18(1) | | |
| Ile | 0.95(1) | 1.84(2) | 1.01(1) | | 2.86(3) | 0.85(1) | 1.93(2) | |
| Leu | | | 1.01(1) | 0.92(1) | 2.83(3) | 1.69(2) | 0.91(1) | |
| Tyr | | | 3.50(4) | 0.12 | 2.84(3) | 1.36(2) | 0.14(1) | |
| Phe | | 0.91(1) | | 0.93(1) | 1.03(1) | 0.75(1) | | |
| Lys | | 4.12(4) | 5.86(6) | 0.24 | 5,18(5) | 5.31(5) | 0.13 | 1.07(1) |
| His | | 1.03(1) | 1.02(1) | | | | | |
| Arg | | | 0.89(1) | | 0.84(1) | | 0.88(1) | |
| Trp | | | + (1) | | | | | |
| Total | 8 | 17 | 41 | 4 | 33 | 24 | 9 | 4 |
| Yield(%) | 34.6 | 39.6 | 27.9 | 35.2 | 19.9 | 8.2 | 8.5 | 20.8 |

Table III-IX. Amino acid compositions of staphylococcal protease peptides of honey bee Cm-cytochrome c. Amino acid analyses were performed on 24-h hydrolysates. Tryptophan was detected by its fluorescence. Values in parentheses were taken from the sequence.

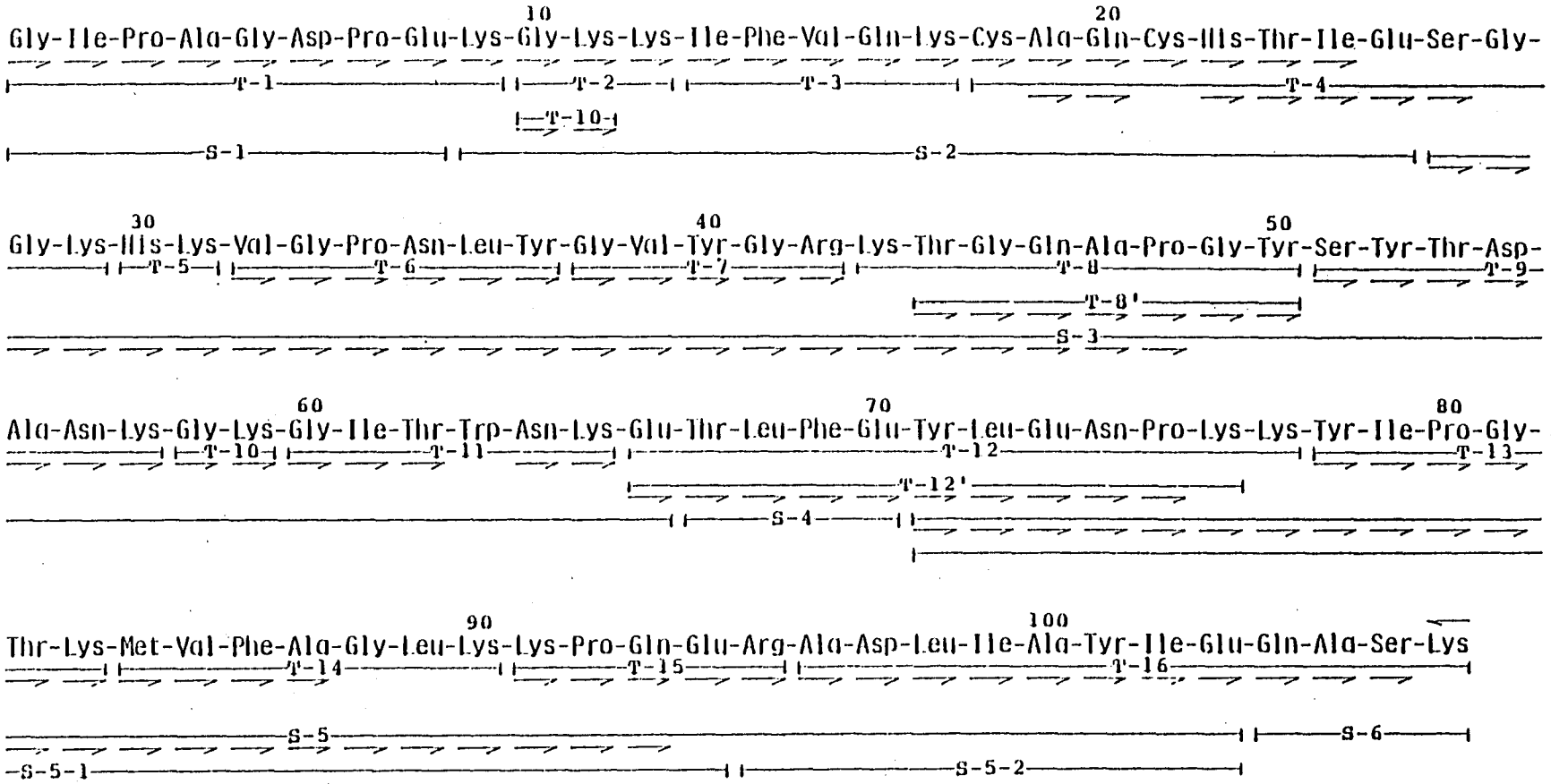


Fig. III-12. Summary of sequence studies of honeybee cytochrome c. T- and S- refer to tryptic and staphylococcal protease peptides, respectively. The amino acid sequence was determined by manual Edman degradation (—>) and carboxypeptidase digestion (<—). Cys was determined as Cm-cysteine.

Tyr-37, Tyr-50, Ile-102, Glu-103 and Ser-106 were specific to honeybee cytochrome c though these residues are conserved among Orthoptera, Lepidoptera and Diptera. Honeybee cytochrome c has Lys-17 which corresponds to Lys-13 of mammalian cytochrome c and is known to be very important for the cytochrome c molecule to react with cytochrome c oxidase. The residue is replaced by arginine in all other insect cytochromes c so far known. In section IV.3, the evolutional relationships of honeybee cytochrome c to other insect cytochromes c will be further discussed.

# IV. EVOLUTIONAL ASPECT OF INSECT CYTOCHROMES c

## 1. PHYLOGENETIC TREE OF INSECT CYTOCHROMES c

Cytochrome c has been extensively used as a source of amino acid sequence data in the construction of molecular phylogenies. For example, the phylogenetic tree of cytochromes c constructed by Baba et al. [9] is shown in Fig. IV-1. Properties such ubiquity among aerobic eukaryotes, low rate of mutation acceptance and relative ease of purification and sequence determination have made cytochrome c a good choice for such studies.

The auther has already determined sequences of five insect cytochrome c; they are housefly larval-type and adult-type cytochromes c, fruit fly, flesh fly and honeybee cytochromes c. All insect cytochromes c already sequenced are summarized in Fig. IV-2. Fig. IV-3 shows a phylogenetic tree which was constructed on the basis of a minimum base change matrix (Table IV-I) according to Fitch and Margoliash [5]. As discussed in section III.2, the sequences of the fruit fly and screw-worm fly cytochromes c determined by Margoliash and his colleagues and blowfly cytochrome c were suggested to be mistaken. If this assumption is correct, the phylogenetic relationships among fly species in the tree will be replaced with those shown in Fig. III-9(B).

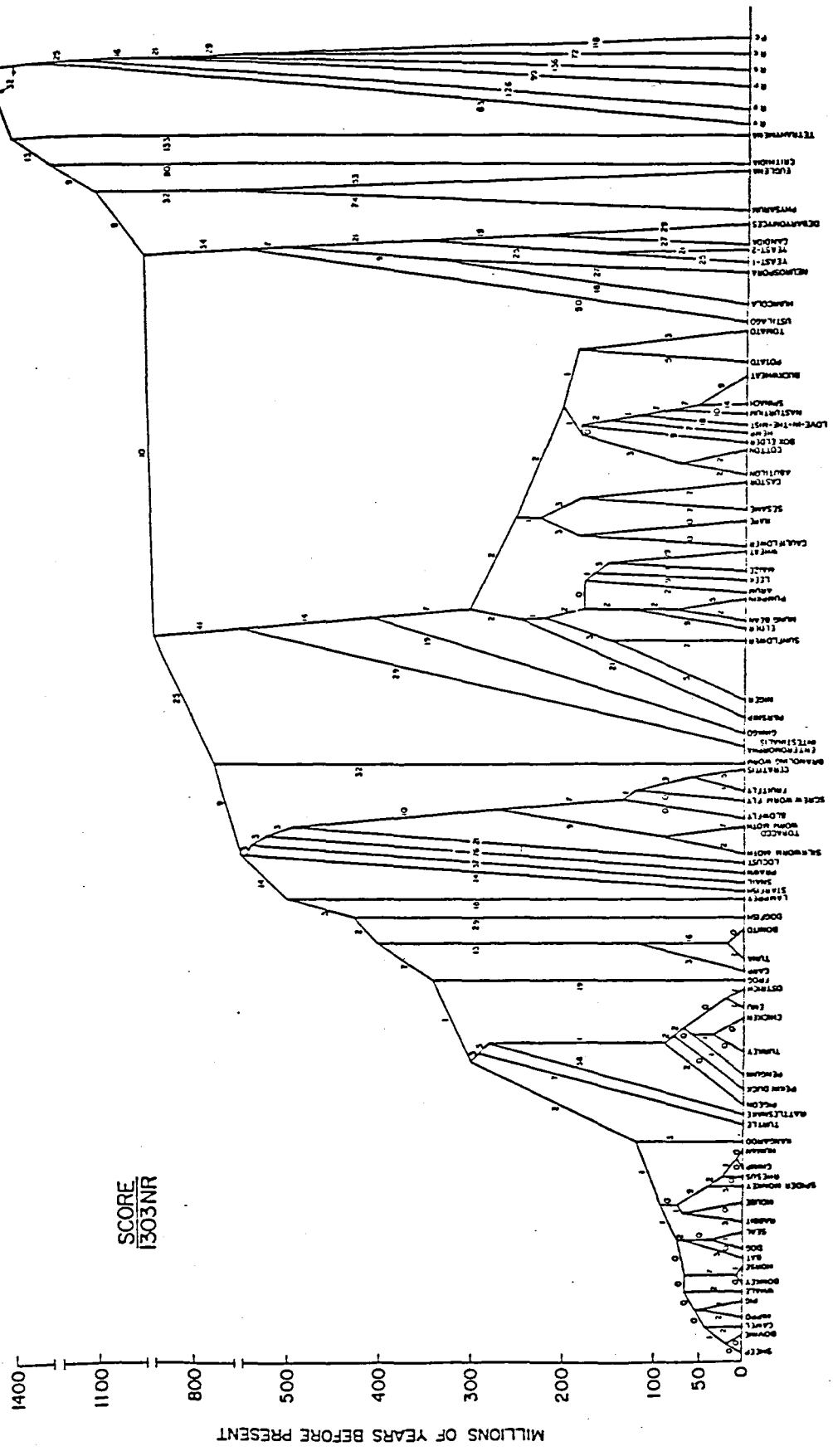


Fig. IV-1. Phylogenetic tree of cytochromes c constructed by Baba et al. [9].

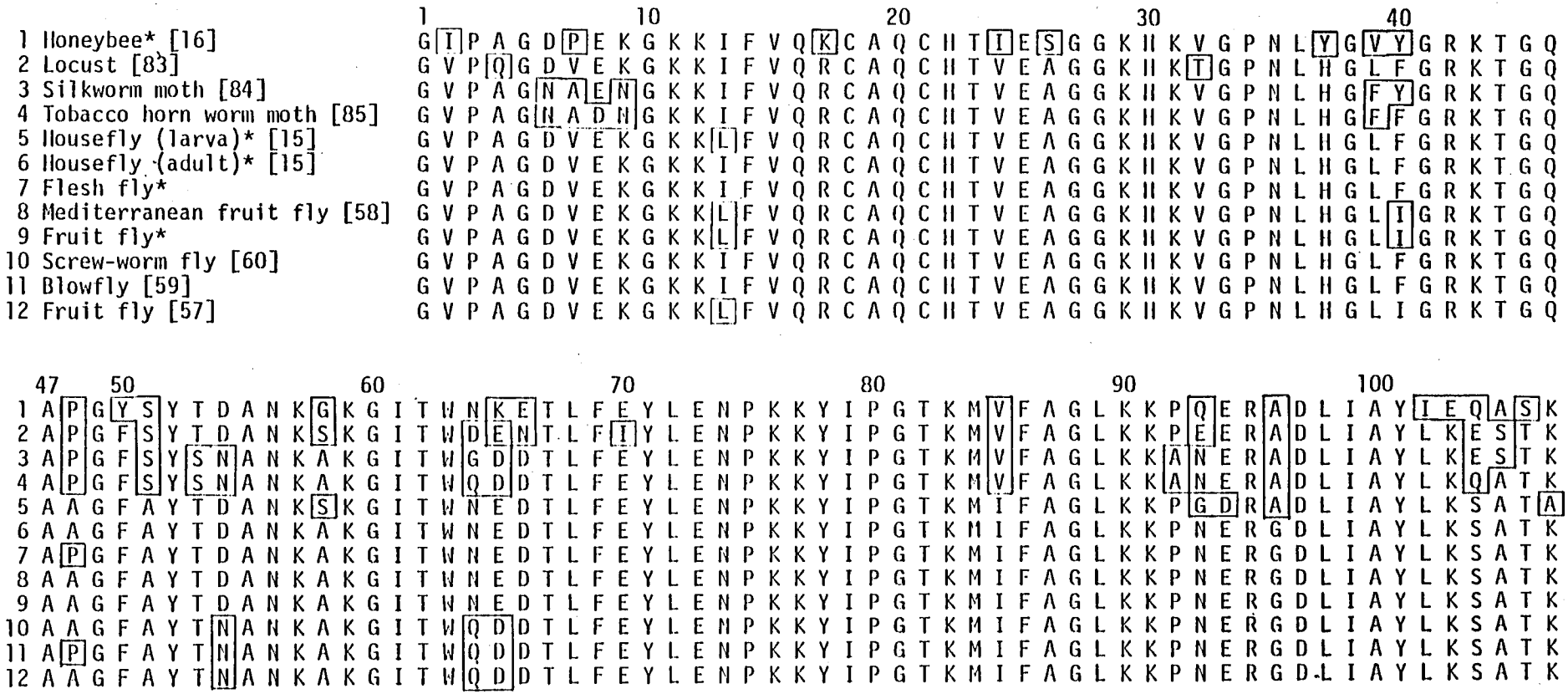


Fig. IV-2. Comparison of amino acid sequences of insect cytochromes c. The boxed residues indicate the amino acids which differ from those of adult-type cytochrome c of the housefly.

* Present study.

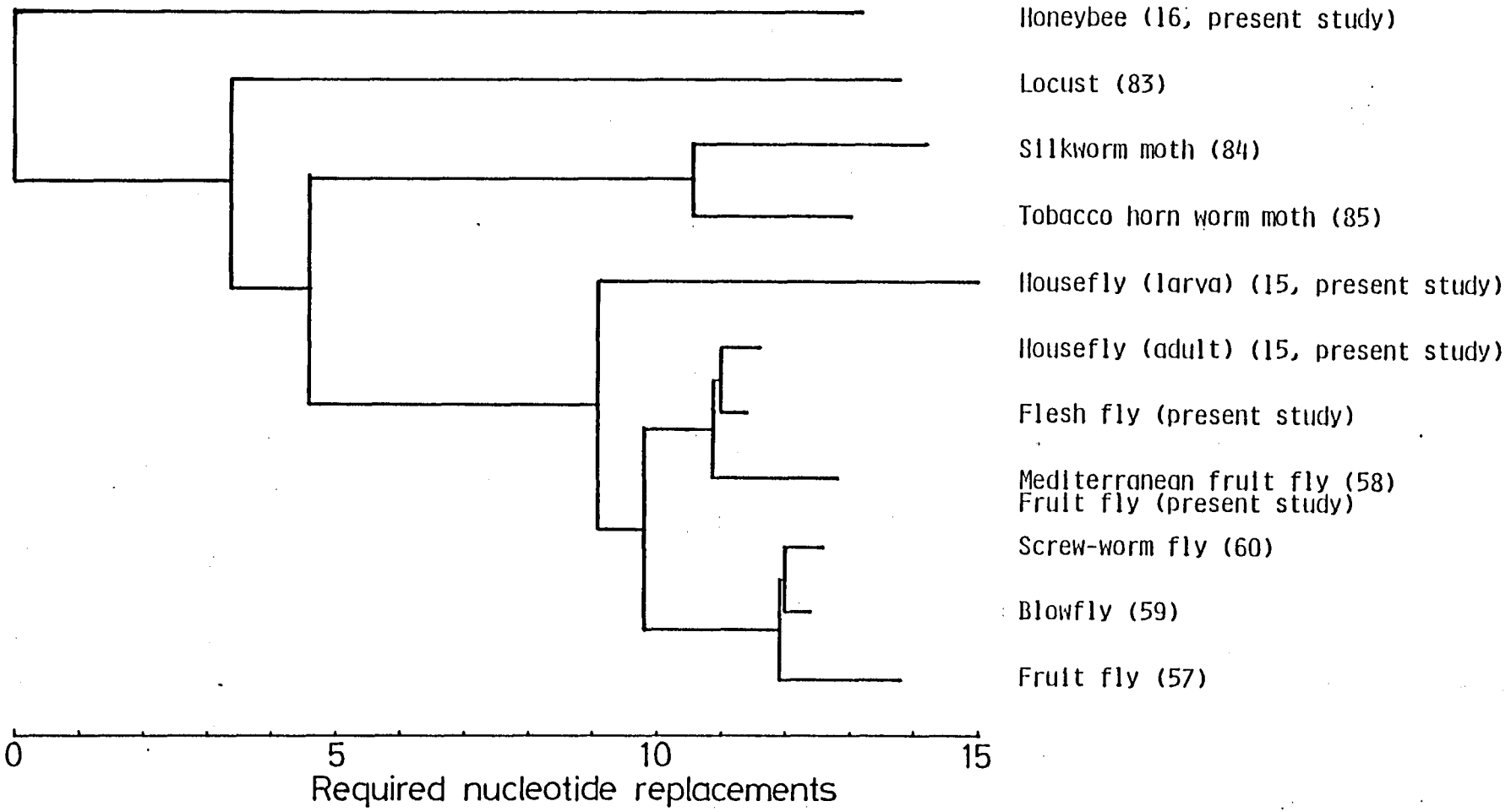


Fig. IV-3, A phylogenetic tree of insect cytochromes c. Branch lengths were calculated on the basis of the matrix prepared from minimum base change shown in Table IV-I.

| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | (Minimum base change) | | | | | |
| (A) Honeybee (16, present study) | | 27 | 26 | 26 | 27 | 24 | 23 | 26 | 28 | 27 | 30 |
| (B) Locust (83) | 22 | | 20 | 23 | 19 | 18 | 17 | 20 | 21 | 20 | 23 |
| (C) Silkworm moth (84) | 23 | 16 | | 6 | 24 | 18 | 17 | 20 | 16 | 15 | 18 |
| (D) Tobacco horn worm moth (85) | 23 | 18 | 5 | | 23 | 17 | 16 | 19 | 13 | 12 | 15 |
| (E) Housefly (larva) (15, present study) | 23 | 14 | 20 | 19 | | 8 | 9 | 8 | 12 | 13 | 12 |
| (F) Housefly (adult) (15, present study) | 21 | 13 | 16 | 15 | 6 | | 1 | 2 | 4 | 5 | 6 |
| (G) Flesh fly (present study) | 20 | 12 | 15 | 14 | 7 | 1 | | 3 | 5 | 4 | 7 |
| (H) Mediterranean fruit fly (58)<br>Fruit fly (present study) | 22 | 15 | 17 | 17 | 6 | 2 | 3 | | 6 | 7 | 4 |
| (I) Screw-worm fly (60) | 23 | 15 | 14 | 12 | 9 | 3 | 4 | 5 | | 1 | 2 |
| (J) Blowfly (59) | 22 | 14 | 13 | 11 | 10 | 4 | 3 | 6 | 1 | | 3 |
| (K) Fruit fly (57) | 24 | 17 | 15 | 14 | 9 | 5 | 6 | 3 | 2 | 3 | |
| | | | | | | (Amino acid difference) | | | | | |

Table IV-I. Amino acid difference and minimum base change matrices of insect cytochrome c.

## 2. GENE DUPLICATION DURING THE EVOLUTION OF FLIES

Two isocytochromes c were occurred only in the housefly among the insect cytochromes c so far investigated. As shown in Table IV-I, 6 amino acid differences are found between larval- and adult-type cytochromes c of the housefly, while 0-3 amino acid differences occur among cytochromes c of various adult flies. The difference between larval- and adult-type cytochromes c of the housefly is enough to suggest that a gene duplication has occurred at least before the divergence of fly species. Larval-type cytochrome c does not occur in the fruit fly or the flesh fly, although the gene which encodes larval-type cytochrome c must exist there. The gene which encodes larval-type cytochrome c may be thought to be a pseudogene in the fruit fly and the flesh fly. Recent studies on the structure of rat cytochrome c gene [61, 62] have shown that 25 different EcoRI restriction endonuclease fragments occur which hybridize with the cloned rat cytochrome c gene and they form gene families. Only one or two of the genes are genetically expressed in rat and the others seem to be unexpressed pseudogenes. Rat cytochrome c gene was also used as a hybridization probe to analyze the genomic DNA of other mammals, such as dog, cow and human. In each mammals, approximately 20-30 hybridizing genomic fragments were observed. Therefore occurrence of a lot of cytochrome c-like sequence is a general feature of mammalian genomes. Three of these cytochrome c-like fragements have been sequenced in rat [62]. They appear to be pseudogenes and to have arisen via an insertion into the

genome of cDNA copy of a cytochrome c mRNA molecule. These results indicate that the increase in cytochrome c-like sequences in mammalian genomes may be due to an increase in pseudogenes, which have arisen via the reverse transcription of mRNA molecules.

In contrast, the chicken contains only one functional cytochrome c gene per haploid genome [63] and the yeast Saccharomyces cerevisiae contains 2 functional genes per haploid genome [64], CYC 1 and CYC 7. These functional genes represent the only cytochrome c-like sequences found in the genome of these 2 species.

Though it is not known whether insect genomes contain many cytochrome c-like genes as observed in mammalian genomes, at least one pseudogene must be present in the fruit fly and the flesh fly. Two models will be thought to explain the evolutional process of larval-type cytochrome c gene.

First model is schematically shown in Fig. IV-4. In this model, larval-type cytochrome c gene is assumed to have been expressed through the evolution of the fly species, and it has later become unexpressed pseudogene in the fruit fly and the flesh fly. Because larval-type cytochrome c of the housefly is thought to be constitutive, and even if it is not expressed by mutation cytochrome c will be supplied by increased amount of adult-type cytochrome c which is thought to be adaptive. As larval-type cytochrome c seems not to be necessarily essential, it may be under weaker selective pressure than adult-type protein. Therefore, it might have become a pseudogene in the fruit fly and the flesh fly after the divergence of these flies.

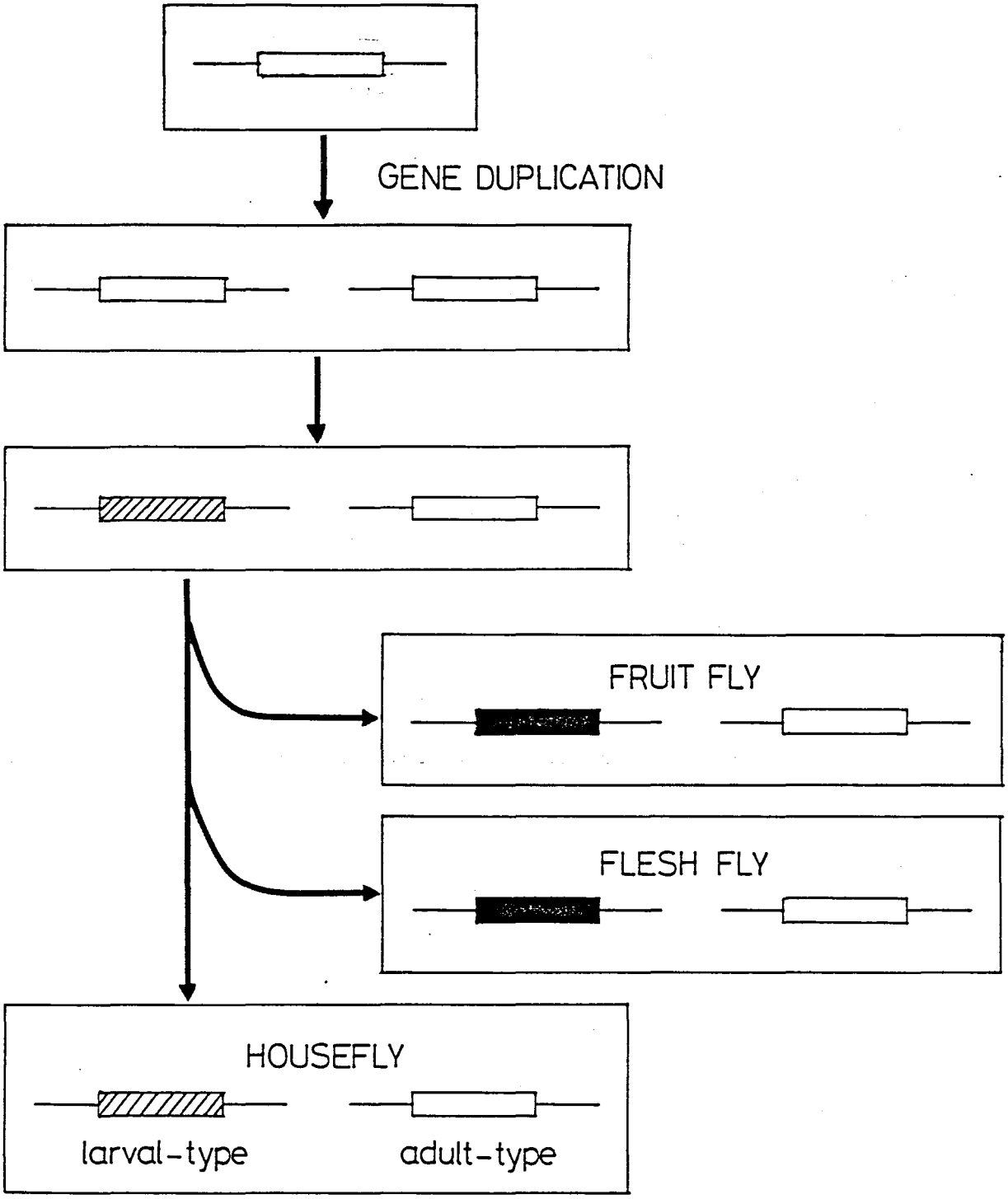


Fig. IV-4. Hypothetical evolution of fly cytochrome c genes (model 1). ▭, expressed gene (regulated); ▨, expressed gene (not regulated); ▬, unexpressed pseudogene.

In this model, the gene duplication may have created two functional gene copies of cytochrome c. Protein encoded by one gene copy might have remained adaptive and that encoded by another copy might have become constitutive through the evolutional process. CYC 1 (the structural gene for iso-1-cytochrome c) and CYC 7 (the structural gene for iso-2-cytochrome c) genes of yeast [65] are good example of the duplicated genes which encode similar proteins with presumably identical functions but exhibit different regulatory responses.

The second model is schematically shown in Fig. IV-5. In this model, the recombination is assumed to have occurred after the divergence of the housefly and larval-type cytochrome c gene is assumed to have been derived from the recombinant gene between a functional gene and a preexisting pseudogene. The recombination has probably occurred, because the amino acid difference between larval- and adult-type cytochromes c are seen mainly in the carboxyl-half of the cytochrome c molecule. The recombination would provide the promotor sequence to the recombinant gene and cause the expression of this gene. If the recombination does not include the regulatory sequences, the way of the gene expression should be constitutive. It was already reported that CYC 1 and CYC 7 genes of yeast had evolved by recombination of each other [66]. In this model, the pseudogene might have originated from the gene which has cytochrome c-like sequences as observed in mammalian genomes. So larval-type cytochróme c gene might not contain the intervening sequence because such cytochrome c-like sequences had arisen via an insertion into the genome of cDNA copy of a mRNA molecule for

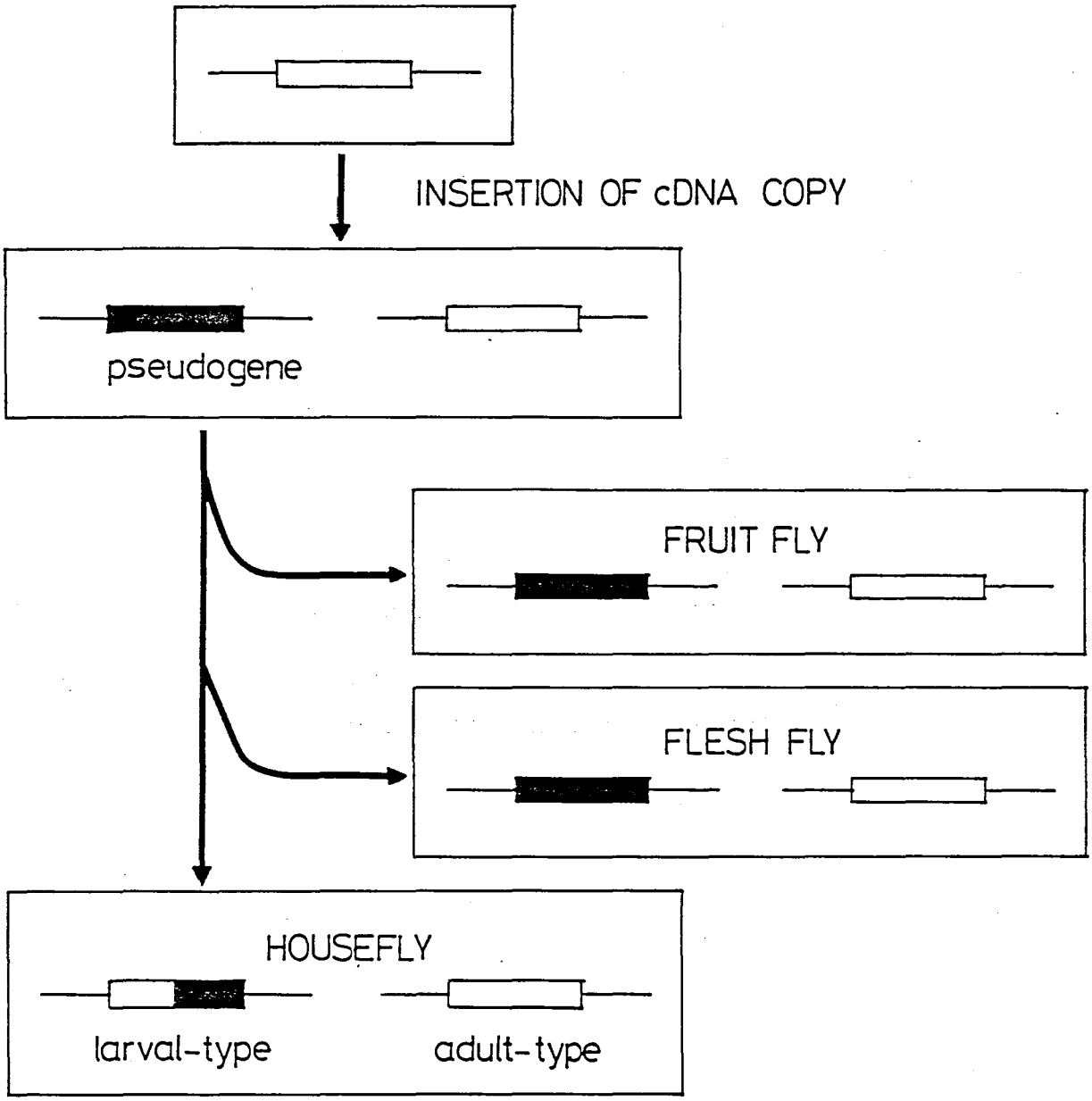


Fig. IV-5. Hypothetical evolution of fly cytochrome c genes (model 2). ▭, expressed gene; ▬, unexpressed pseudogene; ▭▬, the recombinant gene between functional gene and pseudogene.

cytochrome c. The structural genes for cytochrome c in rat [67] and chicken [63] have the intervening sequences in the position that correspond to the residue 60 of the insect cytochrome c molecule but yeast gene for cytochrome c does not contain such sequences [64, 68]. Adult-type cytochrome c gene has probably the intervening sequences. It has been reported that the intervening sequences may contain the presumptive controlling elements that play an important role in the devolopmental expression of globin genes [69]. Then, it might be possible to explain the difference in regulation of larval- and adult-type cytochromes c by the fact whether the genes contain the intervening sequences or not.

Nucleotide sequence studies about larval- and adult-type cytochrome c genes of the housefly will make clear whether the models discussed above are correct or not. It seems that the adult-type cytochrome c mRNA may be easily purified because the adult-type protein increases dramatically at the adult emergence and occupies approximately 1% of the total proteins in the flight muscles.

## 3. EVOLUTIONARY RELATIONSHIP OF THE HONEYBEE TO OTHER INSECTS ON THE BASIS OF THE SEQUENCE

As discribed in section II.3, isocytochromes c do not exist in the honeybee. The phylogenetic tree (Fig. IV-3) supports this results, because the honeybee has diverged before the gene duplication responsible for evolutionary appearance of isocytochromes c in the housefly.

The phylogenetic tree is not consistent with the classical phylogenetic relationships of insect; Hymenoptera, Lepidoptera and Diptera have been classified among Oligoneoptera while Orthoptera has been classified as Polyneoptera. This disagreement is due to the relative large amino acid differences between honeybee and other insects. As shown in Table IV-II, the average amino acid difference between honeybee and other insects is 22.5, while that between locust and other insects excluding honeybee is 15.6. Although the differences between moth cytochromes c and fly cytochromes c are fairly large, they are still smaller than the differences between cytochromes c of honeybee and other insects. The results obtained seem to conflict with the current idea about evolution of insects based on morphological evidence and fossil records that Orthoptera (locust) have appeared in evolution earlier than Hymenoptera (bee). According to Smart [70] (Fig. IV-6), the time in the evolutional divergence between Lepidoptera (moth) and Diptera (fly) was 250 million years ago, that between Orthoptera and Diptera-Lepidoptera 310 million years ago, and that between

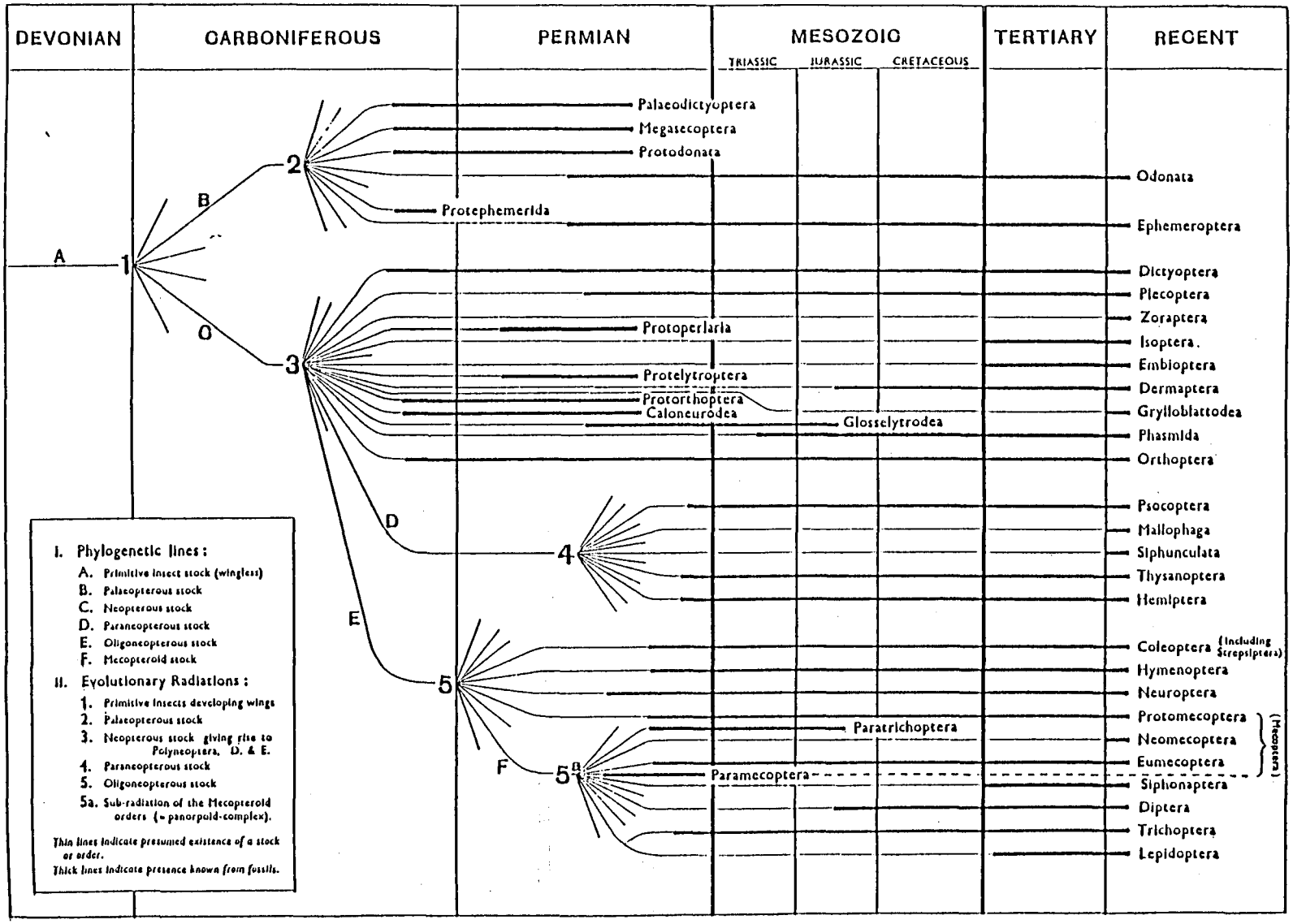


Fig. IV-6. Phylogenetic relationships of insect according to Smart [70].

Hymenoptera and Diptera-Lepidoptera 280 million years ago. If these times in the evolutional divergences of insects as mentioned above are correct, the amino acid difference in the sequence between locust and fly or moth cytochromes c should be larger than that between bee and fly or moth cytochromes c. However, Table IV-I shows that the amino acid differences in the sequence of cytochrome c between honeybee and other insects are always larger than those between locust and other insects excluding honeybee. This may mean that Hymenoptera have appeared in evolution earlier than Orthoptera. However, the fossil records show that Orthoptera have occurred 290 million years ago [70]. These findings may mean that the rate of evolution in terms of amino acid substitution for honeybee cytochrome c differs from those for other cytochromes c.

In Table IV-II, the evolutional rates calculated in terms of amino acid substitution for insect cytochromes c are shown. The rate is expressed by the unit of pauling which means the substitution rate of $10^{-9}$ per amino acid site per year. The evolutional rate between locust and flies, that between locust and moths and that between flies and moths are 0.23, 0.28 and 0.30 pauling, respectively. These values are comparable to 0.3 pauling which has been calculated as the evolutionary rate of cytochrome c on the basis of the sequences for many eukaryotic cytochromes c [71]. The evolutionary rate between honeybee and flies and that between honeybee and moths are 0.41 and 0.43 pauling, respectively, which are evidently larger than the values between other insects described above. The average evolutional rate between bee and fly-moth is about 1.5-fold as fast as that

| Insect groups compared | No. of average amino acid substitution | Time of divergence (years ago) | Evolutional rate (pauling)[a] | Average | Evolutional rate for honeybee (pauling)[b] |
|---|---|---|---|---|---|
| Bee/Fly | 22.0 | $2.8 \times 10^8$ | 0.41 | 0.42 | 0.55 |
| Bee/Moth | 23.0 | $2.8 \times 10^8$ | 0.43 | | 0.59 |
| Locust/Fly | 14.4 | $3.1 \times 10^8$ | 0.23 | 0.27 | |
| Locust/Moth | 17.0 | $3.1 \times 10^8$ | 0.28 | | |
| Fly/Moth | 14.8 | $2.5 \times 10^8$ | 0.30 | | |

Table IV-II. Evolutional rates of cytochromes c among the classes of insects.

[a] For example, the evolutional rate for bee/fly was calculated as follows: $\ln(1 - 22.6/107) \div (2.8 \times 10^8) \div 2 = 0.41 \times 10^{-9}$. The unit of pauling means the substitution rate of $10^{-9}$ per amino acid site per year.

[b] The evolutional rate for honeybee cytochrome c was calculated by assuming that the rate for the cytochrome is faster than those for fly and moth cytochromes c: $0.41 \times 10^{-9} \times 2 - 0.27 \times 10^{-9} = 0.55 \times 10^{-9}$.

between locust and fly-moth or that between fly and moth. In the above, the evolutional rate of honeybee cytochrome c has been calculated by assuming that the same number of the amino acid substitution occurred on both the evolutional lineages of bee and fly-moth since their divergence. However, as the evolutional rate of locust, fly and moth cytochromes c is averagely 0.27 pauling, the rate of honeybee cytochrome c may be calculated to be 0.55 pauling (0.82-0.27). If this value is correct, the evolutional rate in terms of amino acid substitution of honeybee cytochrome c is about twice as fast as that of other eukaryotic cytochromes c. This suggests that the rate of evolutionary change of honeybee cytochrome c may have been more accelerated than that of cytochromes c from other organisms. Such a faster evolutionary rate in honeybee cytochrome c seems to be inconsistent with the neurtal theory of molecular evolution [72,73]; the variation in sequence is brought about by accumulation of point mutations which are neither profitable nor harmful and the rate of variation in sequence is therefore dependent only on the time elapsed since the evolutional branching point. It was already reported by Carlson and Brosemer [44] that cytochrome c might have evolved more rapidly in the honeybee branch than in the other hymenopteran branches on the basis of the amino acid composition of four hymenopteran species, honeybee, bumblebee, leaf-cutting bee and yellow jacket. Faster evolutional change in the sequence of cytochrome c may be attributable to a strong selective pressure brought about by the highly specialized social behaviour. The relationship between insect social behavior and rapid evolution has been suggested also at the anatomical level [74].

# V. REFERENCES

1. Dayhoff, M.O. (1972) Atlas of Protein Sequence and Structure, Vol. 5, National Biomedical Reserch Foundation, Washington, D.C.
2. Dayhoff, M.O. (1973) Atlas of Protein Sequence and Structure, Vol. 5, Supplement 1, National Biomedical Reserch Foundation, Washington, D.C.
3. Dayhoff, M.O. (1976) Atlas of Protein Sequence and Structure, Vol. 5, Supplement 2, National Biomedical Reserch Foundation, Washington, D.C.
4. Dayhoff, M.O. (1978) Atlas of Protein Sequence and Structure, Vol. 5, Supplement 3, National Biomedical Reserch Foundation, Washington, D.C.
5. Fitch, W.M. & Margoliash, E. (1967) Science 155, 279-284
6. MacLaughlin, P.J. & Dayhoff, M.O. (1973) J. Mol. Evol. 2, 99-116
7. Fitch, W.M. (1976) J. Mol. Evol. 8, 13-40
8. Moore, G.W., Goodman, M., Callahan, C., Holmquist, R. & Moise, H. (1976) J. Mol. Biol. 105, 15-38
9. Baba, M.L., Darga, L.L., Goodman, M. & Czelusniak, J. (1981) J. Mol. Evol. 17, 197-213
10. Sherman, F., Taber, H. & Campbell, W. (1965) J. Mol. Biol. 13, 21-39
11. Sels, A.A., Fukuhara, H., Péré, G. & Slonimski, P.P. (1965) Biochim. Biophys. Acta 95, 486-502
12. Hennig, B. (1975) Eur. J. Biochem. 55, 167-185
13. Yamanaka, T., Inoue, S. & Hiroyoshi, T. (1980) J. Biochem. 88, 601-604
14. Yamanaka, T., Inoue, S. & Hiroyoshi, T. (1982) Insect

Biochem 12, 357-359

15. Inoue, S., Hiroyoshi, T., Matsubara, H. & Yamanaka, T. (1984) Biochim. Biophys. Acta 790, 188-195
16. Inoue, S., Matsubara, H. & Yamanaka, T. (1985) J. Biochem., in press
17. Yamanaka, T., Tokuyama, S. & Okunuki, K. (1963) Biochim. Biophys. Acta 77, 592-601
18. Flatmark, T. (1964) Acta Chem. Scand. 18, 1656-1666
19. Broyles, R.H., Pack, B.M., Berger, S. & Dorn, A. (1979) Anal. Biochem. 94, 211-219
20. Weber, K. & Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412
21. Wada, H. & Snell, E.E. (1972) Anal. Biochem. 46, 548-556
22. Spackman, D.H., Stein, W.H. & Moore, S. (1958) Anal. Chem. 30, 1190-1206
23. Williams, K.L., Smith. E., Shaw, D.C. & Birt, L.M. (1972) J. Biol. Chem. 247, 6024-6034
24. Margoliash, E. & Frohwirt, N. (1959) Biochem. J. 71, 570
25. Oakley, B.R., Kirsh, D.R. & Morris, N.R. (1980) Anal. Biochem. 105, 361-363
26. Jacobs, E.E., Andrews, E.C., Cunningham, W. & Crane, F.L. (1966) Biochem. Biophys. Res. Commun. 25, 87-90
27. Merle, P. & Kadenbach, B. (1980) Eur. J. Biochem. 105, 499-507
28. Laemmli, U.K. (1970) Nature 227, 680-685
29. Cabral, F. & Schatz, G. (1979) Methods Enzymol. 56, 602-612
30. Brádford, M.M. (1976) Anal. Biochem. 72, 248-254
31. Yonetani, T. (1961) J. Biol. Chem. 236, 1680-1688
32. Weiss, H., Lorenz, B. & Kleinow, W. (1972) FEBS Lett. 25,

49-51

33. Kadenbach, B., Hartmann, R., Glanville, R. & Buse, G. (1982) FEBS Lett. 138, 236-238

34. Ando, K., Matsubara, H. & Okunuki, K. (1966) Biochim. Biophys. Acta 118, 240-255

35. Broxham, D., Parkelee, C., Kumar, S. & Titani, K. (1982) Biochemistry 21, 2028-2036

36. Shappirio, D.G. & Williams, C.M. (1957) Proc. Roy. Soc. B147, 218-246

37. Chan, S.K. & Margoliash, E. (1966) J. Biol. Chem. 241, 2252-2255

38. Kayser, H. (1984) Z. Naturforsch. 39c, 938-947

39. Levenbook, L. & Williams, C.M. (1956) J. Gen. Physiol. 39, 497-512

40. Herold, R.C. & Borei, H. (1963) Dev. Biol. 8, 67-79

41. Osanai, M. & Rembold, H. (1970) Hoppe Seyler's Z. Physiol. Chem. 351, 643-648

42. Bartelink, A.K.M., Gijzel, W.P., de Kort, C.A.D. & Kroon, A.M. (1975) Dev. Biol. 42, 262-273

43. Herold, R.C. (1965) Dev. Biol. 12, 269-286

44. Carlson, C.W. & Brosemer, R.W. (1973) Syst. Zool. 22, 77-82

45. Eder, J., Kremer, J.P. & Rembold, H. (1983) Comp. Biochem. Physiol. 76B, 703-716

46. Hase, T., Matsubara, H., Kersher, L., Oesterhelt, D., Rao, K.K. & Hall, D.O. (1978) J. Biochem. 83, 1657-1670

47. Wakabayashi, S., Matsubara, H., Kim, C.H. & King, T.E. (1982) J. Biol. Chem. 257, 9335-9344

48. Crestfield, A.M., Moore, S. & Stein, W.H. (1963) J. Biol.

Chem. 238, 622-627

49. Fontana, A., Veronese, F.M. & Boccu, E. (1973) FEBS Lett. 32, 135-138

50. Zimmerman, C.L., Appella, E. & Pisano, J.J. (1977) Anal. Biochem. 77, 569-573

51. Jeppsson, J.O. & Sjöquist, J. (1967) Anal. Biochem. 18, 246-269

52. Edman, P. & Begg, G. (1967) Eur. J. Biochem. 1, 80-91

53. Ambler, R.P. (1972) Methods Enzymol. 25, 262-272

54. Matsubara, H. & Sasaki, R.M. (1968) J. Biol. Chem. 243, 1732-1757

55. Blombäck, B., Blombäck, M., Edman, P. & Hassel, B. (1966) Biochim. Biophys. Acta 115, 1371-1396

56. Laursen, R.A. (1977) Methods Enzymol. 47, 277-288

57. Nolan, C., Weiss, L.J., Adams, J.J. & Margoliash, E., unpublished results, cited by Wojciech, R. & Margoliash, E. (1968) in Handbook of Biochemistry (Sober, H., ed.) pp. C160-C161, Chemical Rubber Co., Cleveland

58. Ferández-Sousa, J.M., Gavilanes, J.G., Municio, A.M., Paredes, J.A., Pérez-Aranda, A. & Rodriguez, R. (1975) Biochim. Biophys. Acta 393, 358-367

59. Shaw, D.C., Williams, K.L., Elizabeth, S. & Birt, L.M. (1978) Biochim. Biophys. Acta 532, 179-186

60. Chan, S.K., Tulloss, I. & Margoliash, E., unpublished results, cited by Dayhoff, M.O. (1972) in Atlas of Protein Seqúence and Structure (Dayhoff, M.O., ed.) Vol. 5, p. D-20, National Biomedical Research Foundation, Washington, D.C.

61. Scarpulla, R.C., Agne, K.M. & Wu, R. (1982) Proc. Natl.

Acad. Sci. USA 79, 739-743

62. Scarpulla, R.C. & Wu, R. (1983) Cell 32, 473-482

63. Limbach, K.J. & Wu, R. (1983) Nucl. Acids Res. 11, 8931-8950

64. Montgomery, D., Leung, D.W., Smith, M., Shalt, P., Faye, G. & Hall, B.D. (1980) Proc. Natl. Acad. Sci. USA 77, 541-545

65. Lez, T.M., Pietras, D.F. & Sherman, F. (1984) Proc. Natl. Acad. Sci. USA 81, 4475-4479

66. Ernst, J.F., Stewart, J.W. & Sherman, F. (1982) J. Mol. Biol. 161, 373-394

67. Scarpulla, R.C., Agne, K.M. & Wu, R. (1981) J. Biol. Chem. 256, 6480-6486

68. Smith, M., Leung, D.W., Gillam, S., Astell, C.R., Montgomery, D.L. & Hall, B.D. (1979) Cell 16, 753-761

69. Schon, E.A., Cleary, M.L., Haynes, J.R. & Lingrel, J.B. (1981) Cell 27, 359-369

70. Smart, J. (1963) Proc. Linn. Soc. London 174, Session 1961-1962 pt. 2, 125-126

71. Dayhoff, M.O. (1972) Atlas of Protein Sequence and Structure, Vol. 5, pp. 50 National Biomedical Reserch Foundation, Washington, D.C.

72. Kimura, M. (1968) Nature 217, 624-626

73. Jukes, T.H. & King, J.L. (1971) Nature 231, 114-115

74. Michener, C.D. (1944) Bull. Am. Mus. Nat. Hist. 82, 151-326

75. Kim, I.C. (1980) Arch. Biochem. Biophys. 203, 519-528

76. Matsubara, H. & Smith, E.L. (1963) J. Biol. Chem. 238, 2732-2753

77. Thompson, R.B., Borden, D., Tarr, G.E. & Margoliash, E. (1978) J. Biol. Chem. 253, 8957-8961

78. Gürtler, L. & Horstmann, H.L. (1970) Eur. J. Biochem. 12, 48-57

79. Walasek, O.F. & Margoliash, E. (1977) J. Biol. Chem. 252, 830-834

80. Scott, W.A. & Mitchell, H.K. (1969) Biochemistry 8, 4282-4289

81. Flatmark, T. & Sletten, K. (1968) J. Biol. Chem. 243, 1623-1629

82. Flatmark, T. (1966) Acta Chem. Scand. 20, 1487-1496

83. Lyddiatt, A. & Boulter, D. (1977) Biochem. J. 163, 333-338

84. Chan, S.K. & Margoliash, E. (1966) J. Biol. Chem. 241, 335-348

85. Chan, S.K. (1970) Biochim. Biophys. Acta 221, 497-501