PART I

CHARACTERIZATION OF TEST SYSTEM MUSCA DOMESTICA AND

EFFECT OF CAFFEINE ON EARLY DEVELOPMENTAL STAGES
INTRODUCTION

Rapid growth and development of larvae of *Musca domestica* represent well-coordinated physiological processes which involve hormonal regulation as in many other insects (Chen, 1971; Doane, 1973; L'Hélias, 1970; Schneiderman and Gilbert, 1964; Thomson, 1975; Wigglesworth, 1970). As such even a minute derangement induced by an environmental agent in the regulatory and synthetic events associated with the growth and metamorphosis of the larvae often reflects in easily-measurable parameters such as growth retardation, decrease in DNA, RNA and protein syntheses and even death (Badmin, 1971; Bergmann et al., 1958; Goyer, 1973; Guerra, 1970; Jakob, 1973; Mitlin, 1956; Mitlin et al., 1954; Reiss, 1975). Furthermore, biochemical analyses of the treated larvae could possibly permit deducing the site of action of the agent.

An attempt has been made here to assess the effectiveness of larval system to measure (indicate) the toxic effects of caffeine which is regarded as a weak mutagen (Malling, 1972) to human beings. It is relevant to point out that the cytotoxic, teratogenic, tumorogenic and mutagenic activity of caffeine is dealt with in several hundred papers (see the review papers of Adler, 1970; Cole, 1971; Deysson, 1968; Epstein, 1970; Kihlman, 1974; Mulvihill, 1973). The data derived from the current experiments reveal that caffeine
exerts very significant adverse effects on the 28-hour-old larvae; these data coupled with the extensive reports in the literature on the biochemical effects of caffeine (Grigg, 1968; Kuhlman et al., 1968; Lieb, 1961; Mitznegg et al., 1971) lend scope for further clarification regarding the target site(s) of action. In this part, these aspects have been discussed.
MATERIALS AND METHODS

The strain of the houseflies used in the present study (*Musca domestica nebulo* Fabr.) has been maintained in our laboratory at $31 \pm 1^\circ C$. The progenitors of this strain consisted of a few pairs of adult flies collected from the campus of this University. The flies were reared on milk-soaked cotton pads and the cotton pads were changed once a day. Other conditions were the same as mentioned by Srinivasan and Kesavan (1977).

For growth studies, population size and rearing media were maintained at near constancy in all work. To insure greater uniformity of the experimental larvae, eggs were collected within a period of 30 minutes from the surface of the cotton pads. The eggs were thoroughly washed with water, disinfected for 10 minutes in 70% alcohol, rinsed with distilled water and incubated on a germination paper at $35 \pm 1^\circ C$. Embryogenesis was also studied at 20, 25 and $30 \pm 1^\circ C$ and time taken for hatching of the larvae was taken as an index. The freshly-hatched larvae were transferred to the beaker containing milk-soaked cotton pads by means of fine brush and allowed to age for different intervals at $35 \pm 1^\circ C$ with a 12 hour light and 12 hour dark cycle. Larval development was also followed at different temperatures.

For caffeine toxicity studies, larvae of 28 hour old were employed as the preliminary experiments have shown
that caffeine is extremely toxic to larvae of less than 24 hour old. Caffeine and other chemicals of desired concentrations were prepared in milk itself in the case of compounds soluble in water. Non-water soluble compounds were dissolved in 0.1N NaOH and suitable aliquot was added to milk to achieve the desired concentration. In control beaker, equal amount of 0.1N NaOH was added. Each beaker received 50 larvae as this number gave optimal growth under the test conditions. Five replicates were prepared for each concentration of chemical and the experiments were carried out at 35 ± 1°C.

Eggs, larvae and pupae were washed with distilled water, dried on a filter paper and weighed on an electro-balance for wet weight. For dry weight, the samples were kept in an oven maintained at 100°C for a period of 6-8 hours and then weighed. Percentage survival and body length of the larvae were recorded at regular intervals. The body length of the larvae was measured by immobilizing the larvae as suggested by Schroeder and Bieber (1971/1972). The measurements were read directly from the scale kept by the side of the larvae. The delay in pupariation of the treated larvae was assessed by recording the pupae formed per unit time. The weight of larvae and pupae were taken as mentioned above. Length of the pupae was measured according to Graf and Benz (1970).
Analytical procedures for DNA, RNA and protein

The sample size taken was around 100 mg. It was homogenized in 0.3M perchloric acid (PCA) (1 ml being used per 20 mg of material). The sample was chilled for at least 10 minutes on ice and the insoluble fraction was collected by centrifugation. The pellet was then extracted with cold 0.3M perchloric acid, ethanol, ethanol-ether (3:1) and ether following the procedure of Linzen and Wyatt (1964). The washed pellet was finally extracted twice with 0.5M PCA for 30 minutes at 70°C. The combined supernatants from the two perchloric acid extractions (70°C) were used for measurement of DNA and RNA. The sediment was solubilized in 1N NaOH at 60°C for 1 hour for protein determination as suggested by Bhargava and Halvorson (1971). DNA was estimated with diphenylamine reagent (Burton, 1956) using calf thymus DNA as standard. RNA was estimated with orcinol (Dische, 1955) using yeast RNA as standard. Protein determinations were carried out by the method of Lowry et al (1951) using bovine serum albumin (BSA) as standard.

Calorific content was determined with a PARR 1412 semimicrobomb calorimeter following the procedure given by Pandian (1969).

Various mathematical formulae have been used by biologists to express the relationship between temperature
and rate of development in insects and other poikilotherms (Davidson, 1944; Phelps and Burrows, 1969). In general, the threshold temperature of development was determined by extrapolating the regression line fitted by eye to the reciprocal values of the developmental times. This method can be criticized since at low temperatures the reciprocal values diverge from the straight line. For that reason, in the present study, the threshold temperature was determined by solving the hyperbolic equation according to Trottier (1971).

**Chemicals**

Yeast RNA, calf thymus DNA, BSA, diphenylamine, orcinol, 5-bromouracil, 5-fluorouracil, hydroxylamine, nicotine, and dimethyl sulfoxide (DMSO) were products of Sigma Co., USA.
RESULTS

In an attempt to characterize *Musca domestica* as a test system, investigations have been undertaken to provide information on its temperature requirements of rapid growth, on the larval stages and length of time necessary for completion of each stage of the life-cycle.

Fig. 1 shows the time taken for the embryonic development of housefly eggs at different temperatures. Notably, as the temperature increases, the time taken for completion of embryogenesis is shortened. The difference of 15°C results in a four-fold reduction on the incubation period. The hatchability percentage at all temperatures remains almost same (90%). The larvae emerged at 35 ± 1°C were fed with milk and were maintained at temperatures ranging from 20 to 35°C. Fig. 2 illustrates the duration taken by the larvae from the time of hatching to reach the puparial stage. With regard to larval development, however, a difference of 15°C results only in three-fold reduction of the duration. The greatest difference is noticeable in hours taken between 20 to 30°C.

Based on data, the hyperbolic equation

\[ \hat{t}_i = \hat{a}(T_i - \hat{b})^{-1} \]

relating the duration of the development of embryo to the onset of hatching (\( t_i \)) at an experimental temperature (\( T_i \)).
was solved by the following method:

Rearrange $t_i = a(T_i - b)^{-1}$ as $t_i T_i - b t_i = a$

Take first differences to eliminate $a$:

$t_1 T_1 - t_2 T_2 = b (t_1 - t_2)$ or $Y_1 = b X_1$

$t_2 T_2 - t_3 T_3 = b (t_2 - t_3)$ or $Y_2 = b X_2$

Embryogenesis:

$$\hat{b} = \frac{\sum_{i=1}^{3} x_i y_i}{\sum_{i=1}^{3} x_i^2} = 14.8$$

and

$$\hat{a} = \frac{1}{4} \sum_{i=1}^{4} t_i (T_i - \hat{b}) = 181.8$$

Therefore $\hat{t}_i = \hat{a} (T_i - \hat{b})^{-1} = 181.8 (T_i - 14.8)^{-1}$

Larval development:

$$\hat{b} = 10.6$$

and

$$\hat{a} = 2274$$

Therefore $\hat{t}_i = \hat{a} (T_i - \hat{b})^{-1} = 2274 (T_i - 10.6)^{-1}$

(For details refer supplement 1 & 2)

The results reveal a higher threshold temperature for embryogenesis than larval development. In this formula $\hat{b}$ is interpreted as the threshold temperature for development, since at a certain minimum temperature of an hyperbolic function the developmental time is infinitely long and growth rate is zero; $\hat{a}$ is interpreted as the minimal accumulated temperature (in excess of $\hat{b}$) required for development.
Fig. 1. Influence of temperature on embryogenesis of *Musca domestica*.

Fig. 2. Effect of temperature on development rate of larvae.

Fig. 3. Changes in wet-weight and dry weight during development.
Supplement for calculating the threshold temperature for development.

I. Embryonic development

Experimental temperatures ($T_i$)  Hours for development ($t_i$)

<table>
<thead>
<tr>
<th>$T_1$</th>
<th>$T_2$</th>
<th>$T_3$</th>
<th>$T_4$</th>
<th>$t_1$</th>
<th>$t_2$</th>
<th>$t_3$</th>
<th>$t_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>25</td>
<td>30</td>
<td>35</td>
<td>35</td>
<td>18</td>
<td>12.5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Solving the hyperbolic equation: $t_4 = \hat{a} (T_1 - \hat{b})^{-1}$

$\hat{b}$ = the threshold temperature for development

$\hat{a}$ = minimal accumulated temperature required for development

Take first differences to eliminate $a$:

$t_1 T_1 - t_2 T_2 = b (t_1 - t_2)$ or $Y_1 = bX_1$

$t_2 T_2 - t_3 T_3 = b (t_2 - t_3)$ or $Y_2 = bX_2$

$t_3 T_3 - t_4 T_4 = b (t_3 - t_4)$ or $Y_3 = bX_3$

$(35 \times 20) - (18 \times 25) = b (35 - 18) \Rightarrow 250 = b(17)$

$(18 \times 25) - (12.5 \times 30) = b (18 - 12.5) \Rightarrow 75 = b(5)\frac{5}{2}$

$(12.5 \times 30) - (8.5 \times 35) = b (12.5 - 8.5) \Rightarrow 77.5 = b(4)$

Then $\hat{b} = \frac{3}{1} \sum x_i Y_i = \frac{3}{1} \sum x_i^2$

$= \frac{(17 \times 250) + (5\frac{5}{2} \times 75) + (4 \times 77.5)}{(17)^2 + (5\frac{5}{2})^2 + (4)^2} = 14.8$

and $\hat{a} = \frac{1}{4} \frac{1}{1} \sum t_i (T_1 - \hat{b})$

$\hat{a} = \frac{35(20 - 14.8) + 18(25 - 14.8) + 12.5(30 - 14.8) + 8.5(35 - 14.8)}{4} = 181.8$

Therefore $t_4 = 181.8 (T_1 - 14.8)$
II. Larval development

Experimental temperatures ($T_1$)  Hours for development ($t_1$)

<table>
<thead>
<tr>
<th>$T_1$</th>
<th>$T_2$</th>
<th>$T_3$</th>
<th>$T_4$</th>
<th>$t_1$</th>
<th>$t_2$</th>
<th>$t_3$</th>
<th>$t_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>25</td>
<td>30</td>
<td>35</td>
<td>240</td>
<td>170</td>
<td>114</td>
<td>90</td>
</tr>
</tbody>
</table>

Other details as mentioned above

$(240 \times 20) - (170 \times 25) = b (240 \times 70) \text{ or } 550 = b (70)$

$(170 \times 25) - (114 \times 30) = b (170 - 114) \text{ or } 830 = b (56)$

$(114 \times 30) - (90 \times 35) = b (114 - 90) \text{ or } 270 = b (24)$

$\hat{b} = \frac{70 \times 550 + (56 \times 830) + 24 \times 270}{(70)^2 + (56)^2 + (24)^2} = 10.62$

$\hat{a} = \frac{240 (20-10.62) + 170 (25-10.62) + 114 (30-10.62) + 90 (35-10.62)}{4}
= 2274$

Therefore $t_1 = 2274 (T_1 - 10.62)$

\[595.772, 547.857, 4\]

\[s^2 = 3.4\]

\[\frac{st}{T} = 8\]
The time course of development has also been followed in terms of wet weight and dry weight of different stages. Further, the relationship between the developmental stage to changes in nucleic acids and protein was also established. Fig. 3 demonstrates the wet weight increase during the development of housefly in which a dramatic increase is seen between 28 hour to 48 hour. The maximum wet weight is registered by the larvae around 70 hour which then declines. The decrease in wet weight is quite sharp around 8 hour and 24 hour after puparium formation. During the middle of the pupal period, an increase in wet weight is also evident, indicating the development of the adult. The freshly emerged male and female adults weigh only about 55% of the maximum larval weight. 0-30 minutes old eggs weigh around 60 μg/egg and freshly hatched larvae around 55 μg/larvae.

The changes in calorific content during embryonic and larval development are given in Figures 4 & 5. Calorific content is 5649 cal/g dry weight in fresh eggs; an initial increase to 5838 cal/g dry weight in 2 hour old eggs is followed by a remarkable decrease during the ensuing stages. The calorific content of the larvae increases till 60-70 hour reaching around 6260 cal/g dry weight. There is a drop in calorific content in the larvae prior to pupariation.
Fig. 4. Calorific content of developing eggs.

Fig. 5. Calorific content of developing larvae.

Fig. 6. Total protein content in eggs during embryonic development.

Fig. 7. Total RNA content in eggs during embryonic development.
Changes in protein and nucleic acids during embryonic development

The results of the determinations of protein quantity during different phases of embryonic development are recorded in Fig. 6. Protein content begins to increase in the first hour and then decline sets in. Around 7th hour, the decrease is about 40% from the original value recorded in 0-30 minutes old eggs.

The quantity of RNA changes drastically during the first hour and shows about 50% decrease after seventh hour incubation (Fig. 7). Contrary to these, DNA shows significant increase during embryogenesis (Fig. 8). Very little DNA (0.6 µg/mg of fresh eggs) is present at the time of oviposition. The peak DNA concentration is 11 µg/mg of eggs, a 18.2x increase in 4 hour old eggs. However, upon emergence of larva, it shows only 13.3x increase.

Since in the present study larval stages of housefly were reared on milk, the results pertaining the RNA, DNA and protein are presented in ratios to dry weight as given by Ring (1973). The development of the larval and pupal stage is referred to, for convenience, in terms of hours from the time of oviposition till adult emergence.
**Protein**

Protein/Dry weight (D.W.) levels fluctuate during larval life showing peaks around 8-9 hour and 24 hour (Fig. 9). The freshly hatched larvae have slightly a higher protein content than eggs. The minimum value is found around 30 hour and then a slow increase is evident in the larval period till puparium formation. In the pupal stage the values show a drop in the first 24 hour corresponding to the histolysis of larval tissues but increase again to reach prepupal level as a result of histogenesis and differentiation of adult tissues.

**RNA**

RNA content can be considered as an index of the capacity of an organism for protein synthesis. Total RNA is, therefore, a measure of the potential rate of protein synthesis. Although, RNA/D.W. levels in the eggs are high, first instar larvae record the maximum (Fig. 10). The levels rapidly decrease in the further larval period by about 60% around 30 hour. There is a slight increase in RNA at 36 and 48 hour, which is followed by a decline. In pupal stage characteristic decrease in RNA values is evident in the initial hours as seen for protein. Newly emerged adults have RNA levels equivalent to that of 80 hour old larvae. The
Fig. 8. DNA levels in eggs during embryonic development.
Fig. 9. Protein/D.W. ratios during development of housefly.
Fig. 10. RNA/D.W. ratios during development of housefly.
Fig. 11. RNA/protein ratios during development of housefly.
RNA/protein ratio curve exhibits fluctuations having peaks around 18 and 30 hour larvae (Fig. 11). Thereafter it shows a steady decline to nearly 10-15% of its peak value. In the middle of the pupal stage, a slight increase is evident.

**DNA**

DNA/D.W. level is very low in the egg but it registers a high value in the just emerged larvae (Fig. 12). Larvae of 36 and 48 hour old exhibit small peaks and the increase is quite marked in the pupal stage. The loss of wet weight associated with pupation is not accompanied by loss of DNA. DNA/protein ratios present a pattern similar to that of DNA/D.W. (Fig. 13).

The RNA/DNA ratio can be regarded as an index of protein synthesis capacity per cell. Since cyclorrhaphan larvae grow by increase in cell size and not by increase in cell number (although Agrell and Lundquist (1973) point out that cell nuclei can increase in size), the fluctuations in this ratio represent variations in protein synthesis capacity per whole animal. The ratios are very high between 30 and 60 hour larval development and a small increase is also observed in pupal stage around
Fig. 12. DNA/D.W. ratios during development of housefly.
Fig. 13. DNA/protein ratios during development of housefly.
Fig. 14. RNA/DNA ratios during development of housefly.
130 hour, reflecting the synthesis of proteins needed for new cuticle and formation of new tissues (Fig. 14). Extremely high values of the RNA/DNA ratio in the eggs indicate the high degree of synthetic activity that takes place during embryogenesis.

The following indices were used for assessing the toxic effect of caffeine on growth and metamorphosis (1) number of larvae surviving at the end of incubation period, (2) length and wet weight of larvae, (3) number, length and wet weight of pupae and (4) number of adults emerged. For comparative analysis of the results with different concentrations and different chemicals some of the above criteria were assigned a numerical value designated as growth index and calculated according to a modified method of Perry and Miller (1965).

\[
(1) \text{Number of larvae recovered} \times \text{wet weight} \left( \frac{\text{mg/larva}}{\text{Number of larvae seeded}} \right) = X
\]

\[
(2) \frac{X(\text{test compound})}{X(\text{control})} = \text{Growth index}
\]

The results obtained on normal growth of the housefly larvae suggest that rapidly proliferating early larvae stages are ideal for toxicological studies. The larvicidal effect was assessed for different concentrations (0.05% - 0.2%) of caffeine. The mortality rate recorded at 24 hour and 40 hour after
treatment is shown in Fig. 15. It seems that caffeine concentration above 0.1% is markedly toxic to the larvae. The effect of caffeine on larval growth, in terms of growth index, is shown in Table 1. These values reflect a linear relationship between dose and effect. The sensitivity of the larval stage was also checked by using a number of chemicals. Of these, methyl xanthines viz., theophylline, aminophylline and theobromine exert an effect quite similar to caffeine. No effect is observed with adenine (Table 2). The halogenated pyrimidines 5-bromouracil and 5-fluorouracil are inhibitory to housefly larval growth (Table 3). With regard to the DNA-binding agents hydroxylamine and spermine, the growth is markedly reduced. Hydroxyurea, an inhibitor of DNA synthesis, is toxic only slightly at higher concentrations (Table 4). Antibiotics streptomycin and terramycin also inhibit the growth of the larvae (Table 5). DMSO and nicotine are extremely toxic to the larvae (Table 6).

Both length and wet weight of the larvae are reduced with increasing concentrations of caffeine (Fig. 16). Interestingly, the length of the larvae following 0.2% caffeine treatment is reduced by about 50% of that of control. On the other hand, with the same concentration the wet weight of the larvae is reduced by about 80% of control.
Fig. 15. Percentage survival of larvae fed on milk containing different concentrations of caffeine. 
a, control; b, 0.05%; c, 0.1%; d, 0.15%; e, 0.2% caffeine.

Fig. 16. Effect of caffeine on the length and weight of larvae. Measurements were taken at the end of 40 hours after treatment. - - - - - length; o-----o weight. The vertical bars at various points represent standard error (S.E.) of the means.
TABLE 1

EFFECT OF INCREASING CONCENTRATIONS OF CAFFEINE ON LARVAL GROWTH OF MUSCA DOMESTICA

<table>
<thead>
<tr>
<th>Caffeine concentration (%)</th>
<th>Growth index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0.05</td>
<td>0.75</td>
</tr>
<tr>
<td>0.07</td>
<td>0.52</td>
</tr>
<tr>
<td>0.10</td>
<td>0.31</td>
</tr>
<tr>
<td>0.15</td>
<td>0.14</td>
</tr>
</tbody>
</table>

The larvae were treated as per procedure laid down in Materials and Methods; the 'Growth index' was computed using the formulae given in Results.
### Table 2

**Effect of Methyl Xanthines (Theophylline, Theobromine and Aminophylline) and Adenine on Larval Growth**

<table>
<thead>
<tr>
<th>Chemical used</th>
<th>Concentration (%)</th>
<th>Growth index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0.05</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.29</td>
</tr>
<tr>
<td>Aminophylline</td>
<td>0.05</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.29</td>
</tr>
<tr>
<td>Theobromine</td>
<td>0.05</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.38</td>
</tr>
<tr>
<td>Adenine</td>
<td>0.10</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>0.78</td>
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</table>

Growth index was calculated as mentioned earlier.
TABLE 3

EFFECT OF HALOGENATED PYRIMIDINES ON LARVAL GROWTH

<table>
<thead>
<tr>
<th>Chemical used</th>
<th>Concentration(%)</th>
<th>Growth index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
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</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>0.29</td>
</tr>
<tr>
<td>5-Bromouracil</td>
<td>0.05</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Growth index was calculated as mentioned earlier.
<table>
<thead>
<tr>
<th>Chemical used</th>
<th>Concentration(%)</th>
<th>Growth index</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>0.05</td>
<td>0.39</td>
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<td></td>
<td>0.10</td>
<td>0.16</td>
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<td></td>
<td>0.20</td>
<td>0.14</td>
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<td>Spermine</td>
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<td>0.85</td>
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<td>0.72</td>
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<td></td>
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<td>Hydroxyurea</td>
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<td></td>
<td>0.10</td>
<td>0.72</td>
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<tr>
<td></td>
<td>0.20</td>
<td>0.72</td>
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Growth index was calculated as mentioned earlier.
TABLE 5

EFFECT OF ANTIBIOTICS STREPTOMYCIN AND TERRAMYCIN ON
LARVAL GROWTH

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration(%)</th>
<th>Growth index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.1</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.12</td>
</tr>
<tr>
<td>Terramycin</td>
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<td>0.42</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>Larvae died</td>
</tr>
</tbody>
</table>

Growth index was calculated as mentioned earlier.
## TABLE 6

EFFECT OF NICOTINE AND DIMETHYL SULFOXIDE ON LARVAL GROWTH

<table>
<thead>
<tr>
<th>Chemical used</th>
<th>Concentration(%)</th>
<th>Growth index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Nicotine</td>
<td>0.005</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
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</tr>
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<td></td>
<td>0.05</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>Larvae died</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
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<td>0.75</td>
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<tr>
<td></td>
<td>1.00</td>
<td>0.66</td>
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</table>

Growth index was calculated as mentioned earlier.
Effect of caffeine on developmental time

Fig. 17 shows the time taken for pupariation of the control larvae as well as those treated with caffeine. The trend of the results seems to indicate that even the lowest concentration of caffeine (0.05%) used perturbs the physiology of growth though it does not cause high mortality. Larval development is severely slowed down at higher concentrations of caffeine; the time taken for 50% pupariation is doubled at 0.1% concentration. Toxicity in terms of percentage puparia formed and also their size, is evident in larvae exposed to caffeine (Table 7 and Fig. 18). The larvae treated with 0.2% caffeine are not able to form puparia at all. The prolongation of development is observed only in larval stage and not in pupal stage. Another point which is evident from Table 7 is the high pupal mortality as judged from adult emergence in the treated population.
Fig. 17. Effect of caffeine on pupariation of housefly.
   a, control; b, 0.05%; c, 0.1%; d, 0.15% caffeine.

Fig. 18. Effect of caffeine on the size of puparia.
   a, control; b, 0.05%; c, 0.01%; d, 0.15% caffeine.
### TABLE 7

EFFECT OF INCREASING CONCENTRATIONS OF CAFFEINE ON THE DEVELOPMENT OF PUPARIA AND PUPAL MORTALITY

<table>
<thead>
<tr>
<th>Caffeine concentration (%)</th>
<th>Length of the puparia (mm ± S.E.*)</th>
<th>Weight of the puparia (mg ± S.E.*)</th>
<th>Percentage of pupal mortality*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.053 ± 0.050</td>
<td>21.405 ± 0.750</td>
<td>10</td>
</tr>
<tr>
<td>0.05</td>
<td>5.311 ± 0.052</td>
<td>14.097 ± 0.730</td>
<td>40</td>
</tr>
<tr>
<td>0.10</td>
<td>5.009 ± 0.058</td>
<td>11.765 ± 0.050</td>
<td>62</td>
</tr>
<tr>
<td>0.15</td>
<td>4.687 ± 0.177</td>
<td>8.958 ± 1.120</td>
<td>80</td>
</tr>
</tbody>
</table>

Larvae of 28 hour old were fed with caffeine containing milk.

* Each value represents the mean of 30-50 pupae with the standard error (S.E.) of the mean.

*Pupal mortality was calculated on the basis of adult emergence.
DISCUSSION

Current trends in bioassay methodology require entire life-cycle tests, including reproduction and testing of the offspring, to detect possible long-term damage induced by toxicants. The rate of development of test animals determines the amount of information that can be obtained in a given time period involving full life-cycle studies. The importance of insect colonies to basic research and practical control has also been augmented by the advances made in the physiology and biochemistry of insect development (Gilbert, 1967; Ilan and Ilan, 1973; Slama et al., 1974; Wilkinson and Brattsten, 1972; Wigglesworth, 1970; Wyatt, 1972). The short life-cycle of *Musca domestica* (about 8 days at 35 ± 1°C) makes it an ideal test organism for full life-cycle studies.

An accurate knowledge of the effect of temperature on the rate of development of *Musca domestica* is a necessary prerequisite for pinpointing specific stages for toxicity studies. The duration of both embryogenesis and larval development was affected by different temperatures (Fig. 1 & 2). These results suggest that temperature 30-35°C supports rapid growth of the larvae.

There is a variety of ways for measuring growth in insects based on physical, physiological and biochemical
parameters (Bursell, 1970; Church and Robertson, 1966; Ring, 1973). In this study, an attempt has been made to characterize the life-cycle of housefly by monitoring wet weight, dry weight, DNA, RNA and protein at different stages of development. Housefly has been exploited extensively in the insect nutrition studies (Galun and Fraenkel, 1957; Levinson and Bergmann, 1957; Monroe, 1962). Compared to other synthetic diets, milk supports the growth of the larvae better and hence milk was used as the medium in these studies.

During embryogenesis, marked changes in total RNA and protein were observed in the eggs. Embryonic development of insects has been studied in a number of organisms (Agrell and Lundquist, 1973; Chen, 1971). Lu and Bodine (1953) determined the changes in DNA-phosphorus and RNA-phosphorus in both the whole eggs and the embryos during egg maturation. They found that RNA-phosphorus increased from 0.94 μg/egg at day 1 to 1.60 μg/egg at the time of hatching (1.7 times). Whereas DNA increased from 0.12 μg/egg to 2.64 μg/egg or 26 times the initial level. Devi et al. (1963) studied the variation in nucleic acid content in various stages of the life-cycle of the confused flour beetle (Tribolium confusum) and reported that RNA synthesis preceded protein synthesis and followed DNA
synthesis in embryogenesis. In the present study, an increase in protein content was observed in 1 hour old eggs with a corresponding decrease of RNA from the original level. The decrease in RNA content may suggest that RNA is used for protein synthesis. Housefly eggs also have a higher RNA/DNA ratio at the time of oviposition suggesting the synthetic capacity of the system (Counce, 1973; Chen, 1971). The nurse cells have been shown to supply the housefly oöcyte with RNA (Bier, 1963; Goodman et al., 1968; Petzelet and Bier, 1970) and cytoplasm (Goodman et al., 1968) as observed in *Drosophila*, *Hyalophora*, *Rhyncosciara*, *Forficula auricularia*, *Labidura riparia* and *Antheraea polyphemus* (Chen, 1971). Ribosomes from housefly eggs have also been shown to occur in monomeric, dimeric and polymeric forms (Gadallah et al., 1970a).

The biological events accompanying embryonic development could be associated either with changes in the levels of enzymes already present or else to the appearance of new enzymes during development. Margulies and Chargaff (1973) and Harris and Forrest (1971) found comparable changes in DNA polymerase and RNA polymerase activity in the course of embryonic development of *Drosophila* and *Onconelius*. In this regard, the enhanced protein content in 1 hr old housefly eggs possibly has some significance.
The decreasing RNA content curve has a peak in 4 hour old eggs (Fig. 7) indicating the synthesis of new RNA. This may correspond to the morphogenetic movements during this period (segmentation and differentiation of organ systems). Blevins (1973) showed that in Aedes aegypti synthesis of RNA is very limited during embryogenesis. Lockshin (1966), too, did not observe incorporation of uridine into RNA in coleopteran egg in the early stages. Detailed studies carried out by Harris and Forrest (1967, '70, '71) on Oncopeltus fasciatus indicate that synthesis of rRNA was first detected during gastrulation stage and it was further confirmed by checking up the template activity of embryonic chromatin from different stages. The decrease in total RNA during embryonic development was also observed by Painter and Kilgore (1967) in housefly eggs. However, RNA content per milligram of eggs reported by these authors is much lower than the values observed in the present studies.

During embryonic development of housefly eggs DNA content showed an increase (Fig. 8). Although Painter and Kilgore (1967) observed a lag period of one hour, there was no evidence of it in the present study. By injection of labeled precursors and autoradiographic analysis,
Lockshin (1966) observed that in coleopteran egg (Leptinotarsa decemlineata and Dermestes maculata) thymidine was incorporated into nuclei at all early developmental stages. Harris and Forrest (1967) studied DNA synthesis in the developing embryos of Oncopeltus and showed that DNA increases rapidly up to gastrulation. Nuclear divisions are also characteristically rapid in housefly eggs as in Drosophila melanogaster and a complete mitotic cycle takes around 8 minutes at 25°C (West et al., 1968). As in many other organisms, the insect egg contains also a large amount of cytoplasmic DNA (Chen, 1971; Lu and Bodine, 1953) and this storage DNA is believed to supply precursors through the action of nucleases for such rapid nuclear multiplications (Muhammed et al., 1967).

The relative changes (Fig. 4) in the calorific content of housefly eggs during embryonic development are similar to those reported for the isopod Ligia oceanica (Pandian, 1972). The steady decrease in the protein content (Fig. 6) parallels the decrease in energy content suggesting that the decrease is partially attributed to the oxidation of the protein yolk, a protein carbohydrate complex which along with lipid yolk is the main form of food reserve during embryogenesis (Chapman, 1969). The protein yolk is most abundant and is usually found in the form of granules described as
"yolk platelets" (Balinsky, 1970). Measurements of the respiratory quotient in a number of insects suggested the utilization of carbohydrate at the beginning of embryogenesis and of fat during a later period (Chen, 1971; Gilbert, 1967). The marked decrease in calorific content observed in 6 hour old eggs could as well be due to the use of lipid as source. During the development of many marine crustaceans, fat serves as the main energy source (Pandian, 1967, '72; Pandian and Schumann, 1967). Upon oxidation, fat releases large quantities of water (1 g fat 1.07 g water; 1 g protein 0.41 g water; 1 g carbohydrate 0.56 g water, Baldwin, (1964). Unlike protein, fat oxidation does not result in ammonia production, the removal of which costs considerable quantities of water, These two properties of fat obviously represent advantages for eggs of terrestrial organisms. Such a view is also evident from the data of Needham (1950) on eggs of terrestrial animals viz., chick (Gallus domesticus), Grasshopper (Melanopus sp), silkworm (Bombyx mori) and sheep blow fly (Lucilia sericata). The energy content of the developing larvae (Fig. 5) coincides with the reported lipid content for Musca larvae (egg 2.7% of fresh weight and larvae 7.1% of fresh weight - Gilbert, 1967). In the beginning of the final instar of Housefly larvae the
accumulation of glycogen is intensified in preparation for metamorphosis and then during pupal stage most of this is consumed for the development of adult (Ludwig et al., 1964).

The analyses of post-embryonic development of *Musca domestica* reveal changes in biochemical parameters. Male and female housefly could not be analysed separately as there is no satisfactory method of separating them until after adult emergence (Painter, 1972). Relationships between growth and biochemical parameters have already been reported by a number of authors: *Aedes aegypti* (Blevins, 1973; Lang et al., 1965), *Anthonomus grandis* Boheman (Vickers and Mitlin, 1965), *Drosophila melanogaster* (Alonso, 1973; Church and Robertson, 1966; Howells, 1972), *Calliphora erythrocephala* Mg (Price, 1965, Sekeri et al., 1968), *Lucilia cuprina* (Howells and Birt, 1966; Lennie et al., 1967), *Gryllus bimaculatus* (Krishnakumaran, 1961), *Tenebrio molitor* (Patterson, 1957) and *Tribolium confusum* (Devi et al., 1963). However, in most studies specific tissues or specific life stages were only selected for analysis. In this study, the various parameters have been determined in whole insects throughout the development (egg to adult) in an effort to define growth so that toxic effect observed with chemicals can be critically looked at.
RNA content can be considered as an index of the capacity of the organism for protein synthesis and DNA content, an estimate of cell number (Blevins, 1973; Ring, 1973). The RNA/DNA ratio is, therefore, an index of protein synthesis capacity per cell (Lang et al., 1965). The decrease in DNA/D.W. ratio in the larval stages could be due to the increase in cell size that occurs during development since it is known that in cyclorrhaphan Diptera larval growth in most tissues is restricted to an increase in cell size and not to an increase in cell number. However, the fluctuations in DNA/D.W. ratio curve between 30 to 60 hour old larvae also suggest the synthesis of DNA.

The results obtained with RNA/D.W. and protein/D.W. ratios reflect cyclic secretion of new cuticle, development of imaginal disks, etc. The highest RNA/DNA ratio was recorded in the egg stage. 40 hour old larvae showed RNA/DNA ratio next to eggs indicating high protein synthesis. In fact the pulse incorporation of injected $2^{-14}$C-glycine into total protein of the silkworm was shown to be parallel to that of RNA/DNA ratio (Tojo, 1971). During the pupal stage which is essentially a closed system, all parameters showed a decline initially coinciding with the histolysis of larval tissues and a rise from the middle of the period when histogenesis and differentiation of
adult tissues occurred. Sivasubramanian et al. (1970) observed in Musca domestica the larval-pupal apolysis and pupal-adult apolysis to occur around 6-8 hour and 40-44 hour respectively after puparium formation at 30 ± 1°C. The changes in RNA values during metamorphosis (Fig. 10) were similar to earlier observations made on other insects (Alonso, 1973; Chinzei and Tojo, 1972; Linzen and Wyatt, 1964; Ring, 1973, Vickers and Mitlin, 1965).

The DNA content observed during the pupal period was very high. The regulation and synchronization of insect development is known to be controlled by the neuroendocrine system (Doane, 1973; Wigglesworth, 1970; Wyatt, 1972). The factors that control the rate of DNA synthesis during larval life, metamorphosis, pupal diapause and adult life in insects were examined by Krishnakumaran et al. (1967). Much information on the influence of hormones on macromolecular syntheses has been obtained in a number of systems (Slama et al., 1974). Another explanation could be the differential extractability of DNA. Howells (1972) showed that the extractability of DNA from Drosophila is different at different developmental stages.

A decade ago, Luning (1966) demonstrated the advantage of an insect system (Drosophila tests) for
assessing the genetic as well as the somatic effects of drugs. The developmental time was chosen as the parameter of somatic effect. The potential toxicity may be expressed as any significant change in the growth of the treated larvae compared to untreated control. In this study, evidences are presented for the somatic effects of caffeine in the housefly larvae by considering more than one parameter. These inter-relating parameters provide the investigator with a wide spectrum to have a closer look at the biological effects of chemicals in a relatively higher system.

From the data presented, it is evident that there exists a linear relationship between caffeine concentration in the medium and observed effects. This is consistent with the suggestion of Luning (1966) that there might be a correlation between the effect on development and survival. The early stage of the larval development was highly sensitive and concentrations above 0.2% were almost 100% lethal. Toxic effect of caffeine is also evident from the data of Howard et al (1975) and Ostertag and Haake (1966) on Drosophila melanogaster larvae. Griffiths and Ducoff (1972) examined the toxicity of various metabolic inhibitors including caffeine in Tribolium castaneum and Tenebrio molitor.
larvae. The maximum sub-lethal concentration of caffeine was 5.0% for short term exposure (4 days) and 1.0% for long term exposure (40 days). It is, however, surprising to note that the larvae of Tribolium could withstand 1% of caffeine. The difference in the medium employed for rearing the larvae of Musca and Tribolium may be one of the reasons for its altered sensitivity in addition to the species difference. In this regard, Singh and House (1970a) showed that effects of antimicrobial agents (potassium sorbate and streptomycin) on growth, development and survival of Agria affinis depend upon the concentration of the agents and upon the nutrient level.

Generally, Musca larvae seem to be highly sensitive to toxic agents. The polyene macrolides filipin and flavofungin have larvicidal activity when added to the diet of Musca domestica larvae (Sweeley et al., 1970). Antimetabolites such as 5-fluorouracil, pyrimethamine, 3-aminotriazole, azaserine, mercaptopurine and fluoroacetamide were shown to be powerful growth inhibitors (LaBrecque et al., 1960; Perry and Miller, 1965). Similarly housefly larval growth was found to be severely hampered by the presence of neopyrithiamine, desoxypyridoxine, 4-aminopteroyl glutamic acid, pantothene, pantoyltaurine, 3-acetylpyridine, 3-aminopyridine, 4-aminopyridine, and picolinic acid.
pyridine-3-sulphonic acid, benzimidazole and p-aminoacetophenone and to a lesser extent by avidine, isonicotinic acid and p-aminobenzene sulphonamide (Levinson and Bergmann, 1959).

The data on growth index obtained with different chemicals (Table 1-6) are qualitatively similar to the agents on Drosophila melanogaster, Musca domestica, Tenebrio molitor, Periplaneta americana and Bombyx mori (Bhattacharyya et al., 1954; Goldsmith et al., 1950; Hinton, 1952; Mitlin et al., 1954; Sieburth and McLaren, 1953; Shyamala and Bhat, 1958). Streptomycin was toxic to the larvae even at 0.1% concentration and terramycin was proved to be extremely toxic (Table 5). Streptomycin has several active groups and may therefore act at different cell levels and one of the most important mechanisms seems to be misreading in protein synthesis (Pestka, 1971). The high toxicity of terramycin seems to indicate that it is resorbed in the gut and diffuses more quickly to the tissues and into the cells. It is of interest to point out that caffeine also gets distributed to various tissues depending on their water content (Burg and Werner, 1972). It is known that tetracyclins interfere with the synthesis of ribosomes (Laskin, 1967). Suppression of the synthesis of ribosomes by 5-fluorouracil also inhibited the growth
of *Drosophila* larvae (Jenkins and Simmons, 1968). The halogenated pyrimidines suppressed the growth of housefly larvae also (Table 3).

Caffeine is known for its pleotropic effects at cellular level: (1) inhibition of cAMP phosphodiesterase (Butcher and Sutherland, 1962), (2) release of membrane bound calcium (Weber, 1968), and (3) inhibition of macromolecular syntheses (Kuhlman *et al.*, 1968; Mitznegg *et al.*, 1971; Putrament *et al.*, 1972; Zuk and Swietlinska, 1973). There are also evidences to indicate that caffeine binds to single-stranded DNA (Domon *et al.*, 1970), enters into complex formation with the blood serum albumin and γ-globulin fractions, haemoglobin, cytochrome c, and various metal porphyrins (Barry *et al.*, 1973; Bender, 1969) and interferes with cellular energy metabolism (Bender, 1969; Dmitrieva *et al.*, 1973). With regard to the effect of caffeine on energy metabolism, Nath and Rebhun (1976) reported that ATP level was not disturbed in sea urchin eggs by caffeine. However, inhibition of respiration and a reduction in NADP and NADPH levels were noted. Some of these mechanisms may contribute to the observed growth reduction of housefly larvae by caffeine.

At higher concentrations, caffeine was lethal and at lower concentrations it interfered with various cellular
functions resulting in the prolongation of growth and development. Levinson and Bergmann (1957) showed that some of the steroids like cholesteryl chloride, 7-oxocholesteryl chloride, Cholestan-3-one and cholestan-3α-ol suppressed larval growth and pupation of *Musca domestica*. Digitonin also decreased growth and rate of pupation of *Musca* larvae proportionally to its concentration. Graf and Benz (1970) observed a similar prolongation of development in *Drosophila melanogaster* larvae treated with streptomycin and terramycin.

The size of the larvae was greatly reduced after treatment with caffeine (Fig. 16). Singh and House (1970b) reported that *Agria affinis* reared on antimicrobial containing diets resulted in abnormally small-sized larvae and the size was inversely proportional to the concentration of the antibiotic.

The apparent growth reduction induced by caffeine could be due to the inhibition of macromolecular syntheses (discussed in more detail in Part III). The experiments on the larvae of different ages (28, 38 and 50 hour old) indicated that caffeine effect is more pronounced at the early stages of the larvae (discussed in more detail in Part II). It is of interest to point out that the stage of the larvae (which has been employed for toxicity studies
here has high transcriptional and translational capacity (Fig. 9, 10 & 14). Such an interpretation is also consistent with that of Mitznegg et al. (1971) who have shown that caffeine effect is marked on proliferating cells. The studies of Wanick (1971) on regenerating liver cells are in accord with this concept. Periasamy and Srinivasan (1974) studied the effect of caffeine on hydra regeneration. Caffeine added immediately after amputation inhibited hypostome regeneration.

Also, the inhibition of phosphogluconate pathway by caffeine may affect the functioning of the cell via reduction of cellular SH-groups and also inhibition of the generation of D-ribose for nucleic acid synthesis (Dimitrov et al., 1969). Another possibility is that caffeine could bring about damaging effect by acting on cell membranes. Caffeine effect on lysosomal membranes has been shown in cultured heart cells by using a sensitive cytochemical technique (Acosta and Anuforo, 1976). Caffeine effect on plant cell membranes also is cleared demonstrated (discussed in detail in Part IV). Sweeley et al. (1970) observed growth retardation and larvicidal effect of filipin (membrane affecting agent) on larvae of Musca domestica, Nauphoeta cinerea and Acheta domestica.

Further, caffeine blocked pupariation completely at 0.2% concentration and lower doses induced considerable
delay in the process which is dependent on the normal functioning of the central nervous system. This could be due to the dysfunction induced by caffeine on brain as the studies of McDaniel and Berry (1974), McDaniel et al. (1976) and Johnson et al. (1976), seem to suggest that methyl xanthines disrupt the normal activity of the brain. Blaustein and Schneiderman (1960) reported that caffeine specifically attacked the insect central nervous system causing extensive degenerative changes in the brain and segmental ganglia. The studies of Yu and Terriere (1974) demonstrated that compounds which enhance or inhibit the activity of microsomal oxidases, exert a considerable pupariation delay and other lesions which are typical of hormone imbalance. It is to be pointed out that caffeine has been shown to enhance the activity of microsomal oxidases (Mitoma et al., 1969). Kirk et al (1971) reported that Drosophila larvae reared on aflatoxin or methylcholanthrene or Rous sarcoma virus containing medium exhibited prolongation of certain phases of insect growth and development. They have suggested that the marked prolongation of the third instar may be due to a severe disturbance in their endocrine balance due to: (i) the affected tissues may be unable to respond to the molting hormone (ecdysone), (ii) the lesions themselves may have caused the prothoracic glands to reduce their
production of ecdysone and/or (iii) the production of prothoracotrophic hormone from the brain may have been upset. The delay in development and prevention of molting was also observed in milkweed bug nymphs and in larvae of *Aedes atropalpus* treated with mycotoxin kojic acid (Beard and Walton, 1969).

The pupa obtained from caffeine treated larvae were small suggesting that caffeine may also affect the maintenance metabolism of the larvae (Fig. 18 and Table 7). Clark *et al.* (1961) showed that pupae and adults of *Pectinophora gossypiella* were about one-third of normal size on diets containing butaben and methylparaben. Several antimicrobial substances have been reported to have detrimental effects on the insect when added in the medium (Kishaba *et al.*, 1968; Moore *et al.*, 1967; Ouye, 1962; Prokopy, 1967).

The ability of an animal to survive exposure to a potentially toxic substance often stands or falls on its capacity first to detoxify and subsequently to remove the material from its body (Wilkinson and Brattsten, 1972). It is evident from the results that ingested caffeine is not detoxified completely in the larval system of the housefly. This has resulted in a considerable percentage of pupal mortality (Table 7), like that of 'exotoxin' of
*Bacillus thuringiensis* where the dose-mortality curves for larval and total mortality of *Drosophila* did not overlap (Perron and Benz, 1968). Indeed, the studies of McDaniel and Berry (1973) have shown the persistence of caffeine in the haemolymph of diapausing pupae and pharate adults of *Hyalophora cecropia*. Further, the work of Blaustein and Schneiderman (1960) on the saturniid moth *Callosamia promethea* and of McDaniel and Berry (1974) on *Hyalophora cecropia* has shown that caffeine and other methyl xanthines inhibit adult development.

Being a purine derivative, caffeine may mimic normal metabolites and may interfere with cellular control mechanisms. It is to be pointed out that purine analogues (6-mercaptopurine, 6-thioguanine and 8-azaguanine) are not *per se* toxic to cells but become toxic only after being converted to nucleotides. These nucleotides then are thought to cause pseudo-feedback-inhibition of purine synthesis (Elliott, 1973). The recent studies of Goth and Cleaver (1976) have shown that caffeine enters intracellular purine pool after degradation. This could invoke disturbance of the balance of purine pool sizes, end-product inhibition or cAMP and cGMP control mechanisms resulting in toxic effects. Since the metabolic aspects
of caffeine have not been studied in *Musca* larvae, interpretation on mechanisms of caffeine effect will have to await further studies. The results obtained with a number of chemicals on housefly larvae suggest that they can be used for screening the toxicity of drugs and other potential chemicals. It is hoped that, on the ground of the basic similarity of microsomal metabolism of insects and mammals (Wilkinson and Brattsten, 1972) the studies on insects would certainly complement the data from that of a mammalian system in understanding the biological side effects of a plethora chemicals of both synthetic and biological origin.
SUMMARY

Housefly Musca domestica was characterized to determine the optimum conditions for its growth for conducting bioassays.

1. Development from egg to adult occurred in 8 days at 35°C ± 1°C. Both embryonic and larval development were influenced greatly by temperature, ranging from 20, 25, 30 and 35°C. At lower temperature the duration taken for development was longer.

2. The course of embryonic development exhibited decrease in the protein quantity; 7 hour-old eggs showed 40% reduction from the initial level. Total RNA decreased rapidly in the developing egg, falling to 50% of the initial level. On the other hand, total DNA increased and registered peak value in 4 hour-old eggs.

3. The calorific content of the developing eggs showed a decrease from 5649 cal/g dry weight to 5361 cal/g dry weight indicating the utility of energy reserves during embryonic development. Matured larvae registered high calorific content (6260 cal/g dry weight).

4. Wet weight, dry weight, DNA, RNA and protein were also determined in larval and pupal stages of housefly. Wet weight decreased sharply in early pupal stage suggesting the histolysis of the larval tissues.
5. Protein/D.W. levels showed variations during larval and puparial stages, increased levels being correlated with the synthesis of new cuticle etc., prior to ecdysis and the histogenesis of adult tissues prior to emergence.

6. RNA/D.W. levels were highest in the first instar larvae but declined rapidly during larval development. Sharp increase was also noticed in pupal stage.

7. DNA/D.W. levels were very low in the egg but reached highest level during first instar larvae and then declined during larval development. Puparial stages registered relatively high levels of DNA/D.W. than larval stages.

8. The RNA/DNA ratio was high in the egg and in the larvae between 30-60 hour of development.

9. Toxicity studies were carried out with 28 hour old larvae. Caffeine showed dose-dependent mortality. Toxic effects were also evident in terms of the weight and length of the larvae and puparia.

10. Further, the sensitivity of larval system of *Musca domestica* was evident with the following chemicals: theophylline, theobromine, aminophylline, 5-bromouracil, 5-fluorouracil, hydroxylamine, spermine, streptomycin, terramycin, dimethyl sulfoxide and nicotine. All these chemicals exerted dose-dependent growth reduction of the larvae.

11. Caffeine induced a marked delay in the pupariation of the larvae suggesting an interference on processes mediated by hormones.

12. Caffeine arrested adult development.