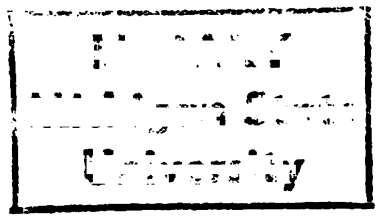

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The Role of 20-Hydroxyecdysone and Dietary Protein in the Regulation of Urate Oxidase Activity During Development of the Third Instar of Drosophila

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Leos G. Kral

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Major professor

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# THE ROLE OF 20-HYDROXYECDYSONE AND DIETARY PROTEIN IN THE REGULATION OF URATE OXIDASE ACTIVITY DURING DEVELOPMENT OF THE THIRD INSTAR LARVA OF DROSOPHILA.

By

Leos George Kral

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ABSTRACT

THE ROLE OF 20-HYDROXYECDYSONE AND DIETARY PROTEIN IN THE REGULATION OF URATE OXIDASE ACTIVITY DURING DEVELOPMENT OF THE THIRD INSTAR LARVA OF DROSOPHILA.

By

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The tissue specific enzyme urate oxidase is confined exclusively to the Malpighian tubules of Drosophila melanogaster and expressed only in the third instar larva and the adult. Shortly before pupariation urate oxidase activity declines precipitously and is not detectable 24 hours later. That 20-hydroxyecdysone is the factor that triggers the disappearance of urate oxidase in the late third instar larvae is demonstrated using the temperature sensitive mutant *ecd*$^{1}$ which at the nonpermissive temperature of 29$^{o}$C fails to accumulate a sufficient concentration of 20-hydroxyecdysone necessary for puparium formation. Both the life cycle and the temporal profile of urate oxidase activity in *ecd*$^{1}$ larvae at 20$^{o}$C is identical to that of the wild type. However, at 29$^{o}$C *ecd*$^{1}$ third instar larvae retain high urate oxidase activity. A precipitous decline in urate oxidase activity is observed when *ecd*$^{1}$ larvae at 29$^{o}$C are fed 20-hydroxyecdysone. These data implicate 20-hydroxyecdysone in the process that controls the rapid decline of urate oxidase activity at the time of puparium formation. Visualization of the urate oxidase peptide in SDS-polyacrylamide gels shows that the amount of the urate

oxidase peptide is correlated with the amount of urate oxidase activity during development. A urate oxidase cDNA clone was isolated from a Malpighian tubule cDNA library and utilized in a Northern gel analysis of mRNA from Drosophila melanogaster larvae at various stages of development. This analysis shows that the amount of urate oxidase activity is correlated with the amount of urate oxidase mRNA present. Rearing third instar larvae on diets containing different amounts of protein and purine compounds shows that urate oxidase activity is primarily induced by dietary protein. The inhibitors of de novo purine biosynthesis, azaserine and DON, inhibit induction of urate oxidase activity by dietary protein.

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## CHAPTER ONE

## Introduction

Urate oxidase activity in Dosophila has been shown to be tissue specific and highly regulated during development (Friedman, 1973). Urate oxidase [E.C.1.7.3.3] converts uric acid to allantoin and is confined to the Malpighian tubules, the excretory organ. Urate oxidase activity is detectable only during the third instar larval stage and in the adult (Figure 1 and Friedman, 1973). No activity is detected during the pupal period. During metamorphosis, many larval tissues in Drosophila are histolized. The Malpighian tubules, however, are not histolized during metamorphosis but are carried over intact to the adult.

In the adult, the reappearance of urate oxidase activity in the Malpighian tubules is synchronized with the emergence of the adult from the puparium. The temporal coordination of the reappearance of urate oxidase activity with emergence has been shown to be controlled in part by an autonomous clock-like mechanism (Friedman and Johnson, 1977; Friedman and Johnson, 1978).

The temporal appearance and disappearance of proteins within a specific cell type during development is presumably due to differential gene expression. If the changes in urate oxiase activity during development of Drosophila can

be demonstrated to be due to differential levels of transcription of the urate oxidase gene, urate oxidase could be a very important model system for the study of complex regulation of eucaryotic genes. To this end, the regulation of urate oxidase during the third larval instar of Drosophila has been studied. Data presented in this dissertation demonstrate that the disapperance of urate oxidase prior to pupariation is dependent on high in vivo levels of the steroid hormone 20-hydroxyecdysone and that the amount of urate oxidase activity during development is correlated with the amount of urate oxidase mRNA.

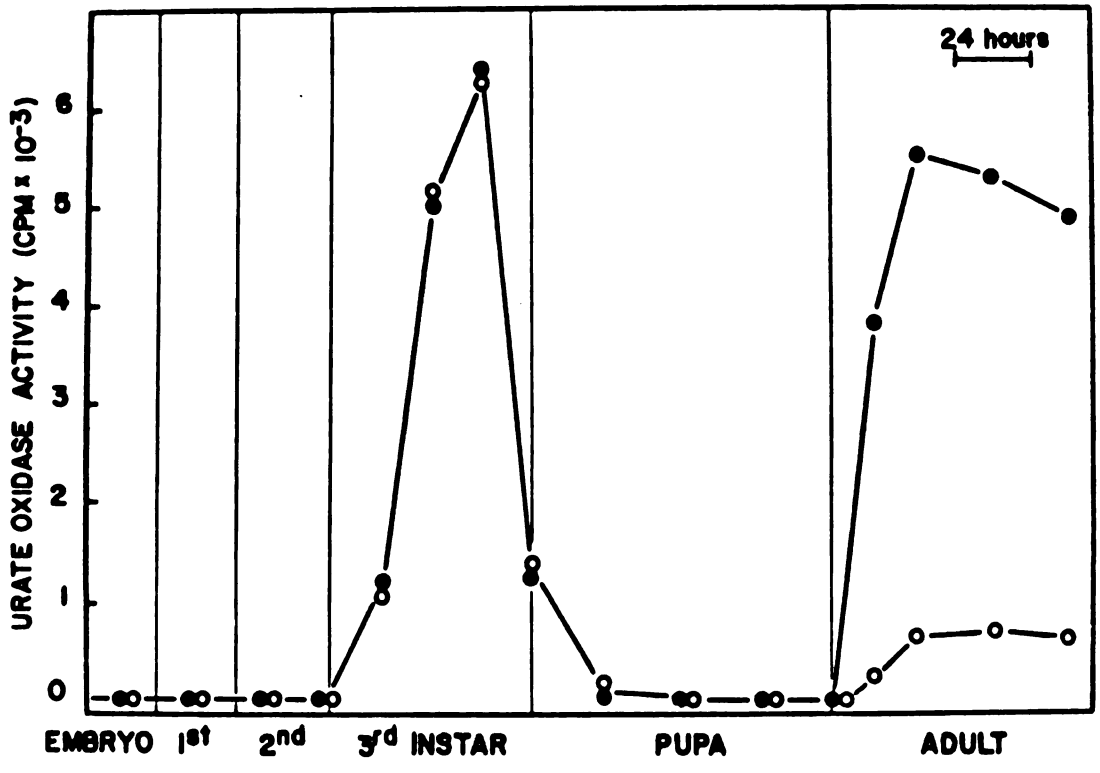


Figure 1. Urate oxidase activity during the development of two strains of Drosophila melanogaster. Theurateoxidase activity of $\underline{ry}^2$: (●) and Ore-R: (○) is expressed as the amount of [$^{14}$C] uric acid converted to [$^{14}$C] allantoin per minute of reaction time per set of Malpighian tubules from a single Drosophila. The bar in the upper right hand corner indicates the time scale at 25°C. The pattern is identical to that previously reported using a spectrophotometric assay for urate oxidase activity (Friedman, 1973).

## Quantitative Regulation of Urate Oxidase

### Demonstartion of the Existence of a Diffusible Urate Oxidase Inducing Factor

As illustrated in Figure 1, the urate oxidase activity of 1-day-old $\underline{ry}^2$ adults is five to tenfold higher than that of 1-day-old Ore-R adults (Friedman, 1973). The data in Table I (Kral et al., 1982) indicate that urate oxidase activity in Malpighian tubules from Ore-R is stimulated by a diffusible hemolymph factor that is present in high concentration in the hemolymph and Malpighian tubules of a newly emerged xanthine dehydrogenase deficient adult. Malpighian tubules from newly eclosed Ore-R adults were transplanted into newly eclosed $\underline{ry}^2$ and Ore-R hosts. After 24 hours, the donor and host tubules were assayed for urate oxidase activity. The Ore-R donor tubules cultured in $\underline{ry}^2$ hosts have on the average 1,683 units of urate oxidase activity, which is approximately four times the activity of the control Ore-R tubules (391 units).

Malpighian tubules from newly eclosed xanthine dehydrogenase deficient adults $\underline{ma\text{-}1;ry}^2$, when transplanted into newly eclosed Ore-R hosts, stimulate urate oxidase activity almost fivefold in the host tubules (Table I). Presumably, the $\underline{ry}^2$ donor Malpighian tubules excrete or release a factor into the blood which then acts upon the Ore-R tubules to stimulate urate oxidase activity. The hemolymph factor which induces urate oxidase appears to be

in higher concentrations in the hemolymph and Malpighian tubules of newly emerged xanthine dehydrogenase deficient adults as compared to the wild-type Ore-R (Table I).

Malpighian tubules from early third instar ecd$^1$ larvae were transplanted into newly eclosed ry$^2$ and Ore-R hosts. After 24 hours of incubation at 25°C the urate oxidase activity of the donor larval tubules as well as the host tubules was determined. As shown in Table II, the urate oxidase activity of the donor larval tubules cultured in ry$^2$ adults is about twofold higher than the activity of donor larval tubules cultured in Ore-R adults. It appears, therefore, that urate oxidase activity is stimulated in larval Malpighian tubules by the same factor that modulates urate oxidase activity in the adult.

A tubule from an early third instar-instar ecd$^1$ larva transplanted into an Ore-R adult induces urate oxidase activity in the Malpighian tubules of the Ore-R adult host (Table II) to the same extent that a tubule from a xanthine dehydrogenase deficient newly emerged adult induces urate oxidase activity in the Malpighian tubules of an Ore-R adult host (Table I). These data indicate that an early third-instar larval Malpighian tubule also contains a considerable quantity of the diffusible factor that induces urate oxidase activity.

**TABLE 1. Urate Oxidase Activity of Adult Donor and Host Malpighian Tubules 24 Hours After Transplantation**

| Genotype of transplanted tubule[b] | Host genotype[b] | Urate oxidase activity[a] | | | |
|---|---|---|---|---|---|
| | | Transplanted tubule | Control for transplanted tubule[c] | Host tubule | Control for host tubule[c] |
| *Ore-r* | *Ore-R* | 655 ± 54.9 | 292 ± 93.0 | 855 ± 253.4 | 292 ± 93.0 |
| *ma-1; ry*$^2$ | *Ore-R* | 2,059 ± 482 | 3,192 ± 834.4 | 1,852 ± 136.5 | 372 ± 64.4 |
| *Ore-R* | *ma-1; ry*$^2$ | 1,683 ± 62.2 | 391 ± 118.8 | 2,590 ± 123.7 | 3,541 ± 188.1 |

[a]Urate oxidase activity is expressed as the counts per minute of ($^{14}$C)allantoin formed from ($^{14}$C)uric acid per minute of reaction time for one set of Malpighian tubules. The numbers are means of two to four independent determinations with three replicas each.

[b]Malpighian tubules to be transplanted were obtained from newly emerged females and transplanted to newly emerged female hosts. The urate oxidase activity in the Malpighian tubules of newly emerged females of both genotypes is essentially zero.

[c]Controls for the transplanted tubules and host tubules represent urate oxidase activity in Malpighian tubules of 24-hour-old female adults of the same genotype as the transplanted tubules and the host tubules, respectively.

**TABLE 2. Urate Oxidase Activity of *ecd*$^1$ Larval and Pupal Donor Malpighian Tubules Cultured in Newly Eclosed Adult Hosts***

| Transplantation protocol | Urate oxidase activity (cpm) | | | |
|---|---|---|---|---|
| | At time of transplantation | | 24 Hours after transplantation | |
| Donor 84-hour-old *ecd*$^1$ larva | Donor: | 293 ± 163 | Donor: | 3,029 ± 1,214 |
| Host: newly eclosed *Ore-R* female | Host: | 70 ± 55 | Host: | 2,124 ± 796 |
| Donor: 84-hour-old *ecd*$^1$ larva | Donor: | 212 ± 198 | Donor: | 6,241 ± 1,085 |
| Host: newly eclosed *ry*$^2$ female | Host: | 268 ± 178 | Host: | 5,916 ± 1,566 |
| Donor: *ecd*$^1$ white pupa | Donor: | 1,421 ± 649 | Donor: | 222 ± 273 |
| Host: newly eclosed *ry*$^2$ female | Host: | 280 ± 169 | Host: | 5,633 ± 1,527 |

The number of independent determinations ranges from 8 to 10.

**ecd*$^1$ larvae were raised at 19°C and Malpighian tubules were transplanted into newly eclosed adult hosts that were maintained at 25°C.

## Temporal Regulation of Larval Urate Oxidase Activity

### Evidence Suggesting a Humoral Repressor

Just prior to pupariation the activity of urate oxidase rapidly declines and is virtually undetectable 24 hours after puparium formation (Figure 1). To probe the mechanism which regulates this decline of urate oxidase activity, Malpighian tubules from *ecd*$^{1}$ white prepupae raised at 20°C were transplanted into newly eclosed *ry*$^{2}$ adults. After 24 hours of *in vivo* incubation the donor pupal tubules were assayed for urate oxidase activity. The data in Table II show that the urate oxidase activity of the donor tubules declined from 1,421 units to 222 units of activity despite the fact that the donor tubules are bathed in the urate oxidase-inducing environment of newly eclosed *ry*$^{2}$ adults. The tubules of the *ry*$^{2}$ host exhibited their normal increase in urate oxidase activity to 5,633 units. These data in Table II indicate that the scheduled decline of urate oxidase activity just prior to pupariation is not simply a reflection of a possible decline in the amount of the urate oxidase-inducing hemolymph factor at puparium formation. Rather, it appears that the Malpighian tubules are no longer competent to respond to the hemolymph factor regulating the quantity of urate oxidase activity.

To test the hypothesis that the loss of competence to respond to the hemolymph factor is an autonomous property of the larval Malpighian tubules, the tubules from early

*ecd*$^1$ third instar larvae were transplanted into newly eclosed *ry*$^2$ adults. The hosts were maintained on rich yeast-honey food at 20°C. The urate oxidase activity of the donor larval tubules and the host tubules was assayed periodically as was the activity of tubules from intact control sister *ecd*$^1$ larvae maintained on normal food at 20°C. In Figure 2, the urate oxidase activity profile of the sister control larvae is superimposed over the urate oxidase activity profile of the *ry*$^2$ host and the transplanted larval tubules. Note that the activity of the transplanted larval tubules closely parallels the activity of the host tubules. The urate oxidase activity of the donor larval tubules, although declining in parallel with the urate oxidase activity of the 84- and 92-hour-old *ry*$^2$ adult hosts, nevertheless remains high at a time when the urate oxidase activity in the tubules of the now pupariating sister *ecd*$^1$ larvae is almost extinguished. One interpretation of the data is that the failure of late third-instar Malpighian tubules to respond to the urate oxidase inducing hemolymph factor (Table II) and perhaps the decline of urate oxidase activity is caused by a factor exogenous to the tubules which acts upon the tubules in the mid to late third instar.

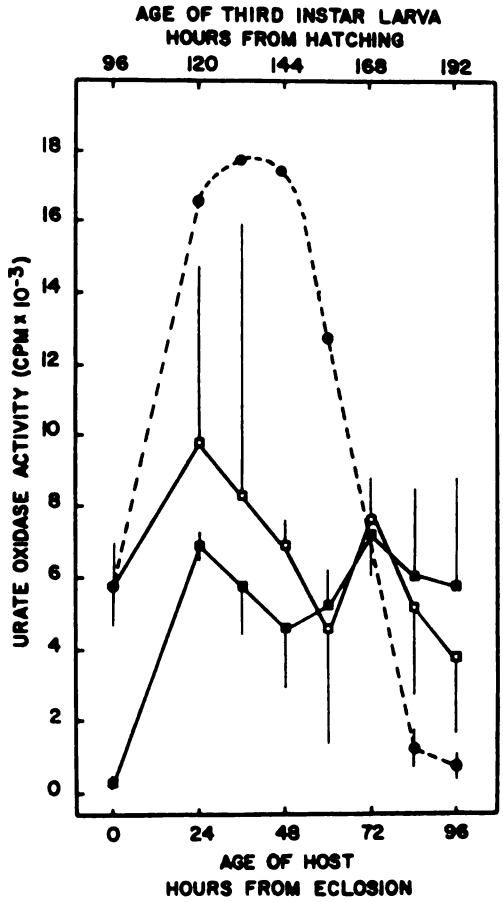


Figure 2. Urate oxidase activity of $\underline{ecd}^1$ larval tubules(●), $\underline{ecd}^1$ larval tubules cultured in $\underline{ry}^2$ newly emerged adults(□), and the $\underline{ry}^2$ host tubules(■). Malpighian tubules from early third instar $\underline{ecd}^1$ larvae were transplanted into $\underline{ry}^2$ newly eclosed adults. Sister larvae and the hosts were maintained on food at 20°C. Urate oxidase activity was determined at times indicated. Each point is an average of four to six independent determinations. Error bars represent standard deviation. For clarity, the error bars were omitted from the from the urate oxidase activity of 120- to 156-hour old larvae( ). The magnitude of the standard deviations at these points is similar to those shown in Figure 3. The mean urate oxidase activity of all $\underline{ecd}^1$ Malpighian tubules cultured in $\underline{ry}^2$ hosts for 96 hours was 6,544 ± 7,024 cpm. This large standard deviation is due to the extremely high activity of one set of Malpighian tubules (20,328 cpm). This data point was eliminated as an outlier ($P < 0.025$) by the statistical test of Grubs and Beck (1972). Therefore, the recalculated mean of urate oxidase activity without the outlier of the $\underline{ecd}^1$ tubules cultured for 96 hours in $\underline{ry}^2$ hosts was 3,788 ± 2,166 cpm.

## Evidence Implicating 20-Hydroxyecdysone as the Repressor

### Effect of the Mutant *ecd*$^1$ on Urate Oxidase Activity

The humoral agent which initiates pupariation in insects is the steroid hormone 20-hydroxyecdysone. The concentration of 20-hydroxyecdysone in the late third-instar larva of Drosophila increases rapidly just prior to puparium formation (Hodgetts et al., 1977; Garen et al., 1977). This increasing titer of 20-hydroxyecdysone coincides temporally with the decline of urate oxidase activity. Because of the correlation of these two events, it was hypothesized that 20-hydroxyecdysone is the exogenous factor which causes the decline of urate oxidase activity in the Malpighian tubules. This hypothesis was first tested by observing how the lack of endogenous 20-hydroxyecdysone in the late third instar larvae of the mutant *ecd*$^1$ (Garen et al., 1977) affects the temporal profile of urate oxidase activity. A similar approach was used by Kraminsky et al. (1980) and Marsh and Wright (1980) to show that 20-hydroxyecdysone induces dopa decarboxylase in Drosophila larvae.

The mutant *ecd*$^1$ isolated by Garen et al. (1977) is phenotypically wild type at the permissive temperature of 20°C. At the nonpermissive temperature of 29°C *ecd*$^1$ larvae fail either to make or accumulate 20-hydroxyecdysone and as a consequence do not form pupae (Garen et al., 1977).

Timed *ecd*$^1$ larvae were raised at 20°C. At 88 hours of postembryonic development, shortly after the second larval

molt, a group of the larvae were shifted up to 29°C. Malpighian tubules from larvae at 20°C and 29°C were assayed periodically for urate oxidase activity. At 20°C the temporal profile of urate oxidase activity in *ecd*$^1$ larvae (Figure 3) is similar to the wild type Ore-R (Figure 1). The activity increases continuously to a maximum and then drops precipitously at the time of puparium formation becoming undetectable shortly thereafter. At 29°C a similar temporal profile of urate oxidase activity is observed in *ecd*$^1$ larvae between 90 hours and 160 hours which includes a partial drop in activity at the time when *ecd*$^1$ larvae form puparia at the permissive temperature (Figure 3). However, the urate oxidase activity of *ecd*$^1$ larvae at 29°C is never extinguished but remains relatively high until the death of the third-instar larva after 1-2 weeks. These data suggest a correlation between the presence of a high endogenous concentration of 20-hydroxyecdysone and the disappearance of urate oxidase activity in the larval Malpighian tubules.

The partial drop in urate oxidase activity observed in late third-instar *ecd*$^1$ larvae at 29°C (Figure 3) also temporally coincides with the wandering stage, a period of time during which larvae cease active feeding that precedes pupariation. Although *ecd*$^1$ larvae do not pupariate at 29°C, they do cease active feeding and crawl out of the food onto the sides of the culture flask. Is it possible that the increase and maintenance of high urate oxidase activity in the third-instar larva is dependent upon continuous

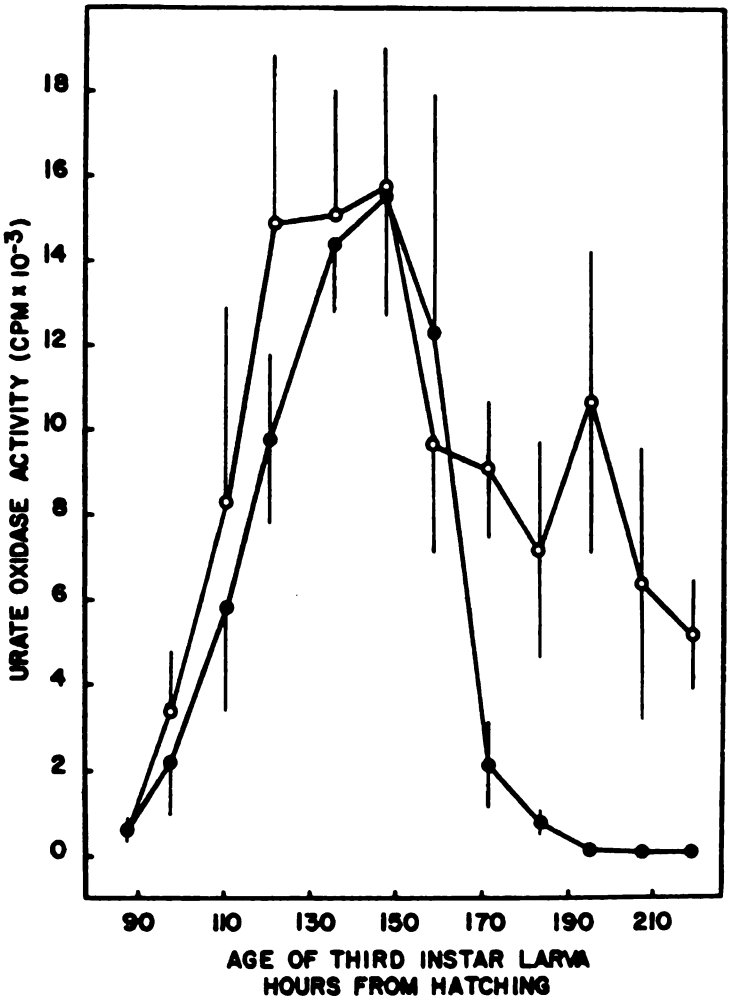


Figure 3. Urate oxidase activity of *ecd*[1] third instar larvae and pupae at 20°C (●) and 29°C (○). Pupariation occurs at 20°C at about 170 hours. Each point is an average of six independent determinations. Error bars indicate standard deviations.

ingestion of food and that the partial loss of urate oxidase activity in *ecd*$^{1}$ larvae at 29°C is due to starvation?

To test this hypothesis, groups of early third instar *ecd*$^{1}$ larvae, maintained on yeast, were shifted from 20°C to 29°C to eliminate endogenous 20-hydroxyecdysone. At the time of temperature shift (Figure 4, arrow b), one group of larvae was transferred from the yeast to inert Whatman CF11 cellulose powder saturated with water. Twelve hours later, (Figure 4, arrow a), another group of larvae was transferred from yeast to cellulose. Urate oxidase activity of all groups was determined periodically. When larvae with high urate oxidase activity are deprived of food, the activity declines (Figure 4, arrow a). When early third-instar larvae with little activity are deprived of food, an increase of urate oxidase activity is not observed (Figure 4, arrow b). Presumably, the amount of hemolymph factor that induces urate oxidase activity in the Malpighian tubules is influenced by the amount of yeast ingested. Continuous ingestion of food appears to be necessary to maintain high urate oxidase activity and explains the partial drop in urate oxidase activity observed in the late third-instar *ecd*$^{1}$ larvae at 29°C (Figure 3). However, even though *ecd*$^{1}$ larvae at 29°C have stopped feeding, relatively high urate oxidase activity persists until death. There is additional discussion of the influence of diet on urate oxidase activity in chapter 2.

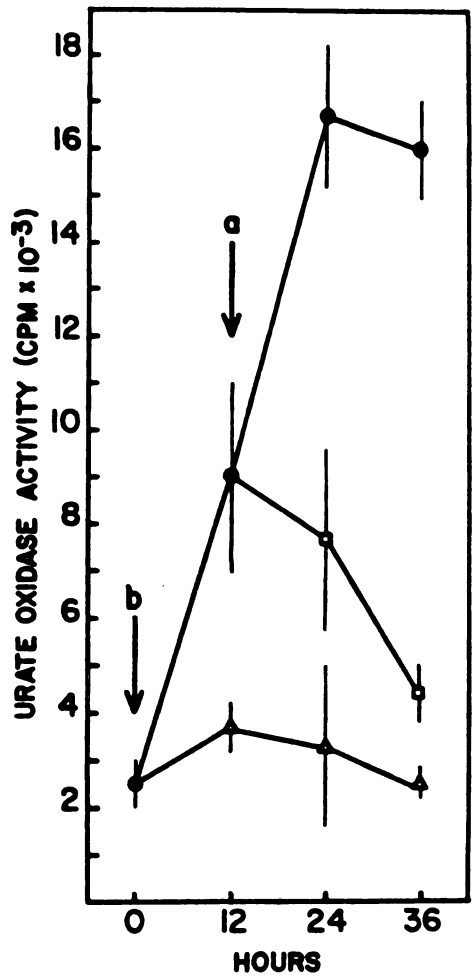


Figure 4. Effect of starvation on urate oxidase activity of third instar ecd[1] larvae. Early third instar ecd[1] larvae were transfered to 10 ml beakers containing yeast paste (●) or moist cellulose powder (△) (arrow b). After 12 hours a portion of the larvae were transfered from the beakers with the yeast to to beakers containing moist cellulose (□) (arrow a). All larvae were maintained at 29°C. Each point is an average of four to six independent determinations. Error bars represent standard deviations.

**Effect of 20-Hydroxyecdysone on Larval Urate Oxidase Activity**

Late third-instar *ecd*$^1$ larvae at 29°C contain no detectable 20-hydroxyecdysone (Garen et al., 1977; Berreur et al., 1984) and also maintain high urate oxidase activity (Figure 3). This suggests that 20-hydroxyecdysone is necessary for the disappearance of urate oxidase activity observed shortly after puparium formation. The primary defect of the *ecd*$^1$ mutation has not yet been determined (Berreur et al., 1984). It is possible that this mutation may affect the temporal regulation of urate oxidase activity by some mechanism that does not involve the titer of 20-hydroxyecdysone. To implicate 20-hydroxyecdysone as the causal agent in the regulatory mechanism responsible for the decline of urate oxidase activity, it is necessary to show that exogenous 20-hydroxyecdysone can trigger the loss of urate oxidase activity in *ecd*$^1$ larvae at 29°C.

Early third instar *ecd*$^1$ larvae were shifted to 29°C to eliminate endogenous 20-hydroxyecdysone. At various times during the maturation of the third instar *ecd*$^1$ larvae at 29°C, groups of larvae were transferred to either yeast with 20-hydroxyecdysone dissolved in ethanol or to yeast and ethanol alone. Urate oxidase activity was assayed at 12-hour intervals. When 112- and 124-hour-old *ecd*$^1$ larvae were fed 20-hydroxyecdysone, a rapid loss of urate oxidase activity was observed (Figure 5, panels C and D). It might be argued that the observed decline of urate oxidase

activity may be a result of the larvae not eating the 20-hydroxyecdysone-supplemented yeast. However, note that the rate at which urate oxidase declines when larvae are starved is much slower (Figure 4) than when larvae of approximately the same age are fed 20-hydroxyecdysone (Figure 5, panel C). These experiments indicate that 20-hydroxyecdysone is necessary for the process that regulates the total loss of urate oxidase activity in the late third-instar larva.

It is interesting that the concentration of 20-hydroxyecdysone (0.4 mM) which triggers the decline of urate oxidase activity when fed to 112- and 124-hour old larvae (Figure 5, panels C and D) does not seem to be immediately effective in preventing the increase in urate oxidase activity when fed to early third-instar larvae shortly after the second larval molt (Figure 5, panels A and B). A similar phenomenon was observed by Lepesant et al. (1978) who were unable to prematurely stimulate the synthesis of 20-hydroxyecdysone inducible fat body P1 protein by feeding 20-hydroxyecdysone to early third-instar larvae.

There are a number of possible explanations for the failure of tissues in very early third-instar larvae to respond to exogenous 20-hydroxyecdysone. The molecular components which mediate the 20-hydroxyecdysone response may not as yet be functional. For example, the 20-hydroxyecdysone receptor protein (Maroy et al., 1978; Yund et al., 1978) may not be present in sufficient quantity in some tissues of the early third-instar larva.

Alternatively, the 20-hydroxyecdysone response may be suppressed by another humoral agent such as juvenile hormone which might be present in the early third instar larva just after the second larval molt. Chihara and Fristrom (1973) have reported that juvenile hormone can inhibit the 20-hydroxyecdysone induced evagination of imaginal discs in vitro as well as the 20-hydroxyecdysone induced [$^3$H] uridine incorporation into imaginal disc cells. The endogenous levels of juvenile hormone during the development of Drosophila have not been published. However, Baehr et al. (1979) have shown that in Locusta migratora a high concentration of juvenile hormone is present during the early part of the last larval instar. If a high concentration of juvenile hormone is present in the early third-instar Drosophila larva, then the feeding of 20-hydroxyecdysone to early third instar larvae may not be capable of prematurely inducing or repressing those 20-hydroxyecdysone dependent functions which are inhibited by juvenile hormone.

It is possible to prevent the scheduled increase of urate oxidase activity in early third-instar larvae by feeding the larvae 20-hydroxyecdysone at concentrations much higher than is necessary to initiate a decline of urate oxidase activity in older larvae (Figure 5, panel E). These data are of questionable significance since concentrations of 20-hydroxyecdysone higher than 1 to 10 mM were lethal to the larvae within 24 hours after feeding. Furthermore, it

is impossible to conclude from these data (Figure 5, panel E) whether the putative inhibitory effect of juvenile hormone was overcome or whether a much higher concentration of 20-hydroxyecdysone is needed at a time when the intracellular concentration of 20-hydroxyecdysone receptors may be very low.

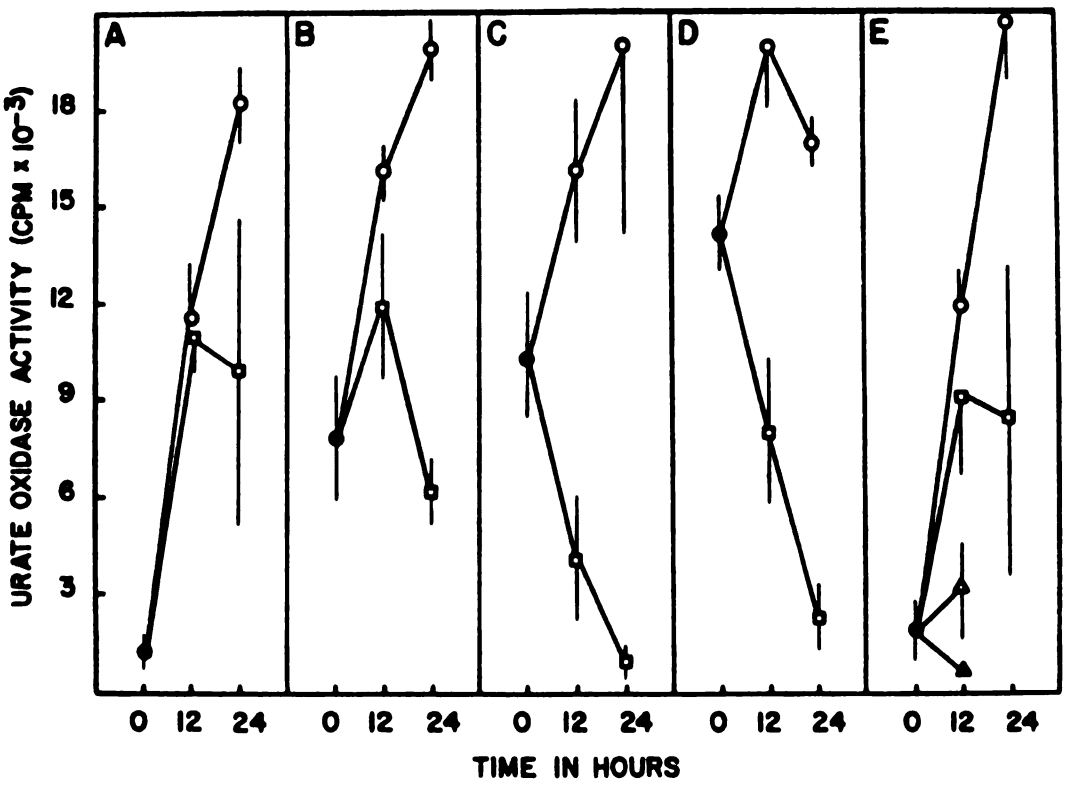


Figure 5. Effect of feeding 20-hydroxyecdysone to *ecd*$^{1}$ third instar larvae at 29°C. 1) At the initial time (●) larvae of approximate age 88 hours (panel A), 100 hours (panel B), 112 hours (panel C), and 124 hours (panel D) were transferred to 10-ml beakers containing yeast paste with (□) and without (○) 0.4 mM 20-hydroxyecdysone. Urate oxidase activity was determined at the time of transfer. 2) Panel E: 88 hour old *ecd*$^{1}$ larvae (●) were transferred to 10-ml beakers containing yeast with no 20-hydroxyecdysone (●), 0.4mM 20-hydroxyecdysone (□), 1.6 mM 20-hydroxyecdysone (△), and 8 mM 20-hydroxyecdysone (▲). Each point is an average of four to six independent determinations. Error bars represent standard deviations.

## Analysis of the Level of Urate Oxidase Regulation

### Temporal Distribution of Malpighian Tubule Proteins

Variations in the amount of enzyme activity *in vivo* can be regulated in a number of ways. A particular enzyme may remain at the same concentration at all times but, 1) its activity can be modulated allosterically or 2) it can exist as an inactive proenzyme which is converted to active form as needed. Alternatively, change in the amount of enzyme activity may be a direct reflection of the number of enzyme molecules.

To test the hypothesis that the change in urate oxidase activity during development of Drosophila is due to a change in the concentration of the urate oxidase protein present in the Malpighian tubules, proteins from Malpighian tubules of *ecd*$^{1}$ early and mid third-instar larvae and white prepupae were fractionated on SDS-polyacrylamide slab gels. These gels show that the pattern of proteins in the Malpighian tubules is highly conserved during the third instar. The majority of proteins of the Malpighian tubules show no change in concentration between early and late third instar (Figure 6). The major exception to this observation is a protein band with an apparent molecular weight of 41,000 which comigrates with homogeneously pure Drosophila urate oxidase (Figure 6, lane 2). The changes in the relative amounts of this 41,000-dalton protein as judged by the density of Coomassie Blue staining correlate with the

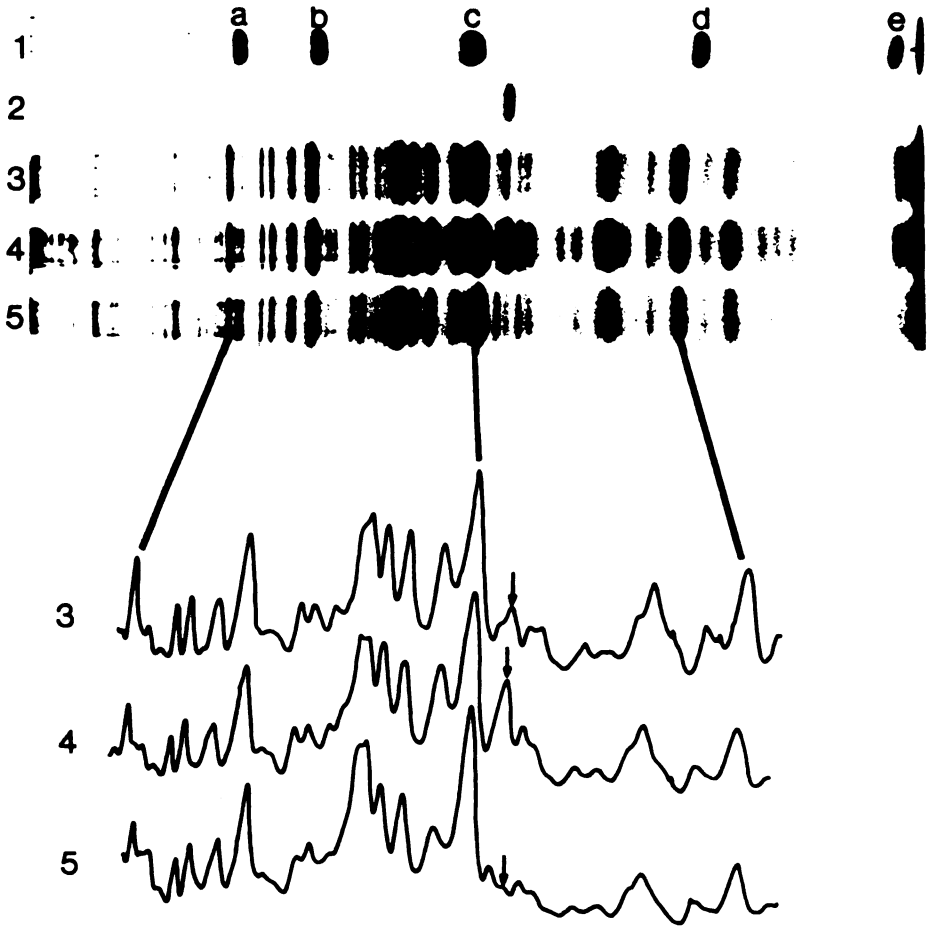


Figure 6. SDS-polyacrylamide gel electrophoresis of Coomassie Blue stained proteins from Malpighian tubules of ecd[1] third instar larvae. Lane 3 contains 30 tubules from 95 hour old larvae, lane 4 contains 15 tubules from 130 hour old larvae, and lane 5 contains 20 tubules from 170 hour old larvae. Lanes 3, 4, and 5 contained approximately equal quantities of protein. Pharmacia low molecular weight standards and purified Drosophila urate oxidase are in lanes 1 and 2 respectively. The molecular weights of the standards are a) 94,000; b) 67,000; c) 43,000; d) 30,000; and e) 20,100. The urate oxidase band in the spectrophotometric scans of lanes 3, 4, and 5 is indicated by an arrow.

changes of urate oxidase activity during the third instar (compare Figure 3 and Figure 6). In the early third instar, when urate oxidase activity is low, a relatively light staining band is present which comigrates with the purified urate oxides polypeptide. During the mid third instar, when urate oxidase activity is at its peak, the 41,000-dalton protein band is densely stained and is one of the major proteins in the Malpighian tubules. At pupariation, when urate oxidase activity is very low, the 41,000-dalton protein band is hardly visible. A direct correlation between urate oxidase activity and the density of the 41,000-dalton protein band that comigrates with purified urate oxidase is observed throughout development. The same correlation is observed in adult Drosophila (data not shown). In Ore-R adults urate oxidase activity is low and the staining of the 41,000 dalton protein band is light. In 24-hour-old $ry^2$ adults, urate oxidase activity is relatively high (Figure 1) and the 41,000-dalton protein band stains correspondingly dark (data not shown).

The majority of the 41,000-dalton protein band (Figure 6) from high urate activity Malpighian tubules is recognized by rabbit anti-Drosophila urate oxidase antibody. A homogenate of $ecd^1$ larval Malpighian tubules with high urate oxidase activity was prepared and equal aliquots were added to preimmune IgG and anti-Drosophila urate oxidase antibody respectively. As shown in Table III, the rabbit anti-urate oxidase IgG inactivated more than 95% of the urate oxidase

Table III. Immunoprecipitation of urate oxidase activity from *ecd*[1] larval Malpighian tubule homogenates.

| Type of rabbit IgG | Percent of urate oxidase activity before | after addition of IgG |
|---|---|---|
| preimmune | 100 | 90.6 |
| immune | 100 | 3.5 |

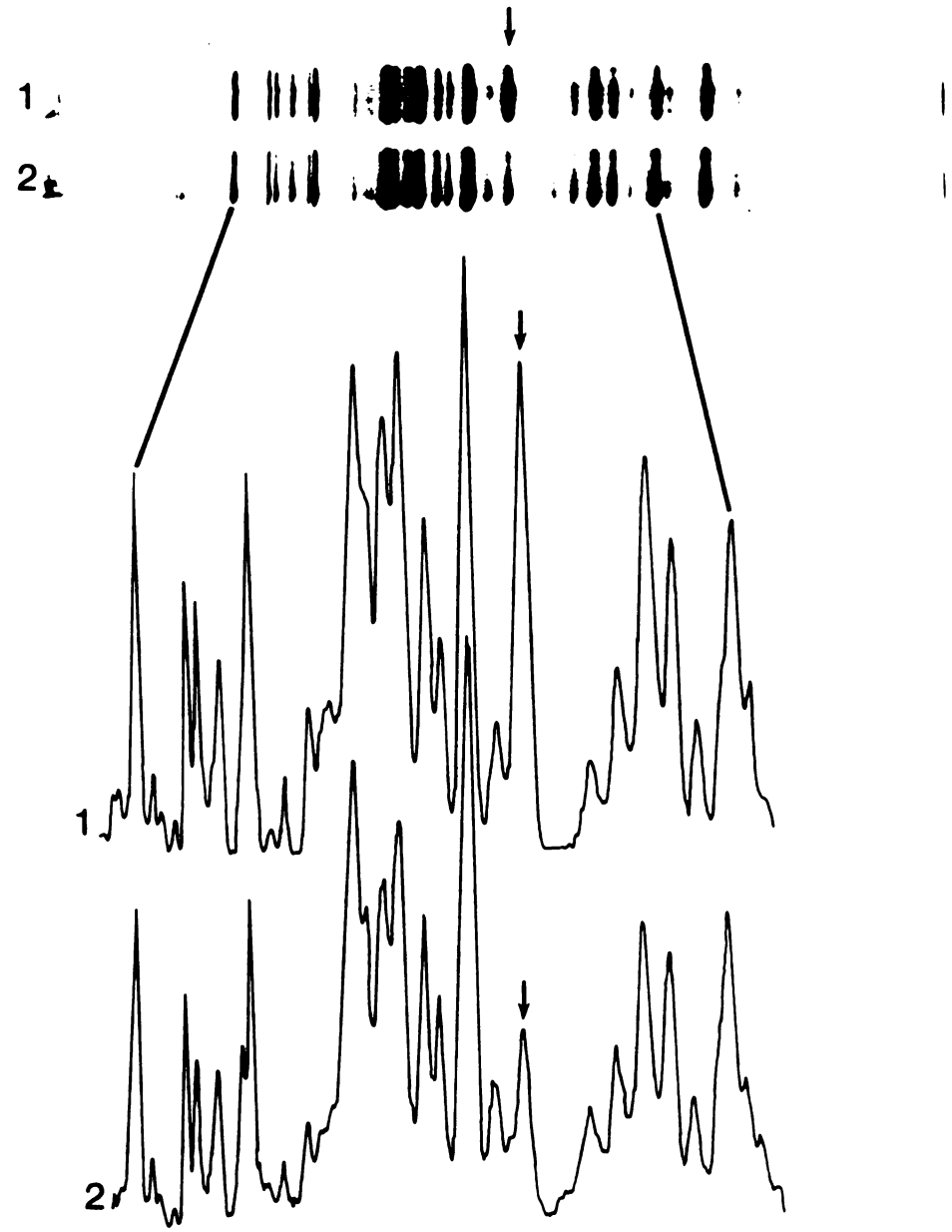


Figure 7. SDS-polyacrylamide gel electrophoresis of silver stained proteins from Malpighian tubules after removal of proteins bound to preimmune IgG (lane 1) and anti-Drosophila urate oxidase IgG (lane 2). Spectrophotometric scans 1 and 2 are of gel lanes 1 and 2 respectively. The protein band that comigrates with purified urate oxidase is indicated by an arrow.

activity, whereas no significant loss of activity occurred in the presence of control preimmune rabbit IgG.

After removal of all IgG and IgG-Drosophila protein complexes by protein A-Sepharose, the remaining Malpighian tubule proteins were fractionated by SDS-polyacrylamide gel electrophoresis and stained with silver. As shown in Figure 7, more than 60% of the 41,000-dalton protein band in the control lane has been removed with rabbit anti-urate oxidase IgG.

The data presented in Figure 3, Figure 6, and Figure 7 show that the amount of urate oxidase activity is a reflection of the amount of urate oxidase protein. Therefore, at pupariation, 20-hydroxyecdysone is involved in regulating the rapid decrease of the concentration of urate oxidase protein in Malpighian tubules.

Steroid hormone mediated repression of the synthesis of some proteins is well documented. Exposure of hepatoma cells to the steroid hormone analogue dexamethasone causes the induction of the synthesis of tyrosine aminotransferase as well as the cesation of synthesis of a number of functionally unidentified proteins (Ivarie and O'Farrell, 1978). Roberts (1975) treated a Drosophila cell line with 20-hydroxyecdysone and reported the cesation of synthesis of several soluble proteins detected by immunoelectrophoresis. Using single dimensional SDS electrophoresis Savakis and coworkers (1980) occasionally detected reduced amounts of a 56,000-dalton and a 79,000-dalton protein in a 20-

hydroxyecdysone treated Drosophila cell line. Coudere and Destugue (1975) also using cultured Drosophila cells identified two peptides whose synthesis appears to be inhibited by 20-hydroxyecdysone. There is, therefore, precedent for steroid hormone mediated cessation of synthesis of several functionally unidentified proteins in Drosophila and other organisms. Urate oxidase appears to belong to this small class of proteins whose synthesis is restricted in the presence of a high *in vivo* titer of 20-hydroxyecdysone.

**Temporal Distribution of Urate Oxidase mRNA**

The magnitude of urate oxidase activity is directly proportional to the amount of the urate oxidase enzyme present in the Malpighian tubules. This implies two possible modes of regulation. First, the amount of urate oxidase mRNA could remain constant and some kind of post - translational control mechanism would regulate the amount of active urate oxidase. Alternatively, the amount of urate oxidase could be directly proportional to the amount of urate oxidase mRNA.

To measure the urate oxidase mRNA levels at different stages of larval development, newly hatched $\underline{ecd}^1$ larvae were collected and maintained on yeast at 20°C. At various times during development, larvae (or prepupae) of specific age were collected and polyA$^+$ RNA extracted from them. The mRNA was then fractionated on a denaturing formaldehyde agarose gel and transferred from the gel to a nitrocellulose filter. The RNA on the filter was then hybridized with [$^{32}$P] labeled urate oxidase cDNA. The isolation of a Drosophila urate oxidase cDNA is discussed in detail in chapter three.

As shown in Figure 8, the amounts of urate oxidase mRNA present at various stages of larval development correlate quite well with both urate oxidase activity (Figure 3) and the amounts urate oxidase enzyme present (Figure 6). There is no urate oxidase mRNA present in newly hatched first instar larvae. A miniscule amount of urate oxidase mRNA is present in the second instar larval RNA preparation.

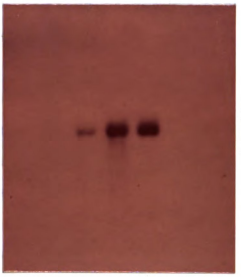


Figure 8. Northern gel analysis of urate oxidase mRNA during the larval stage of Drosophila development. Total RNA was extracted from various stages of larval development. Poly $A^+$ RNA was obtained from 200 ug of each of the total RNA preparations by a single pass through a micro oligo(dT)-cellulose collumn. Each of the poly $A^+$ RNA samples was then denatured in a formamide-formaldehyde buffer and loaded onto a 1.2% denaturing formaldehyde agarose gel. After electrophoresis, the RNA was transfered from the gel to a sheet of 0.45 micron nitrocellulose by blotting. The RNA was then baked on to the nitrocellulose in a vacuum oven for 2 hours at $80^oC$ and the filter was hybridized with $10^7$ CPM (specific activity of 1.6 x $10^8$ CPM/ug) [$^{32}$P]urate oxidase cDNA (pcDNAUO63). After washing the filter was autoradiographed. Only one RNA band hybridized with the urate oxidase cDNA. Each lane contains approximately 5 ug of poly $A^+$ RNA from 1: newly emerged first instar larvae; 2: second instar larvae; 3: 80 hour old third instar larvae; 4: 125 hour old third instar larvae; 5: 145 hour old third instar larvae; and 6: white prepupae. The nitrocellulose was stained with methylene blue after exposure to confirm that equal amounts of poly $A^+$ RNA were transferred from the gel to the filter. (Additional details in the Appendix)

This small amount of urate oxidase mRNA present in the second instar RNA preparation does not necessarily mean that the urate oxidase gene is expressed in this developmental period. The population of second instar larvae may have been contaminated by a few early third instar larvae. Larvae were collected by age in hours from hatching. No attempt was made to visually inspect individual larvae for developmental stage.

The levels of urate oxidase mRNA in third instar larvae (Figure 8) correlates precisely with the third instar levels of urate oxidase peptide (Figure 6). Both the urate oxidase peptide (Figure 6, lane 3) and mRNA (Figure 8, lane 3) are relatively low density bands seen in preparations from early third instar 95-hour-old larvae. During the mid-third instar, both the urate oxidase peptide (Figure 6, lane 4) and urate oxidase mRNA (Figure 8, lanes 4 and 5) are seen as very dense bands. At pupariation, the urate oxidase peptide band is extremely faint (Figure 6, lane 5) and the urate oxidase mRNA is virtually non existent (Figure 8, lane 6).

These results show that urate oxidase activity and the amount of urate oxidase peptide are directly correlated with the level of urate oxidase mRNA. It appears, therefore, that changes in urate oxidase activity are modulated primarily at the level of urate oxidase mRNA transcription and/or post - transcriptional processing.

**Does 20-Hydroxyecdysone Act Directly on the Genome of Malpighian Tubules?**

Data have been presented to show that 20-hydroxyecdysone is implicated in the mechanism by which urate oxidase mRNA, peptide and activity is repressed in the Malpighian tubules of the third larval instar of Drosophila. It has been shown in a number of tissues of Drosophila, including the Malpighian tubules, that 20-hydroxyecdysone acts directly on the genome to regulate gene transcription as evidenced by puffing changes in polytene chromosomes (Ashburner 1967, 1972, 1973,; Bonner and Pardue 1976, 1977; Berendes 1966, 1967). In particular, 20-hydroxyecdysone has been shown to directly repress the transcription of the salivary glue protein gene sgs-4 in Drosophila salivary glands (Beckendorf and Kafatos, 1976; Muskavitch and Hogness, 1980).

To determine whether 20-hydroxyecdysone acts directly on the genome of Malpighian tubules to repress the urate oxidase gene, Malpighian tubules from *ecd*$^{1}$ larvae raised at 29$^{o}$C were cultured *in vitro* in Schneider's medium with and without 20-hydroxyecdysone. In an initial experiment, *ecd*$^{1}$ larvae were raised in the absence of endogenous 20-hydroxyecdysone at 29$^{o}$C on yeast food to a point at which urate oxidase activity peaked. The Malpighian tubules were then dissected from a group of the larvae and each was transferred into 100 ul of Schneider's medium with or without 20-hydroxyecdysone. The cultured tubules as well as

the sister larvae were assayed for urate oxidase activity at 12 hour intervals.

As the results in Figure 9 indicate, urate oxidase activity of the in vitro cultured Malpighian tubules declined at about the same rate as the activity in the tubules of the sister larvae regardless of the presence or absence of 20-hydroxyecdysone in the culture medium. On the average it seems that the tubules cultured *in vitro* in the presence of 20-hydroxyecdysone experienced a greater drop in urate oxidase activity then the tubules cultured in absence of the hormone but 1) the difference is not significant and 2) the rate of decline is not as rapid as that observed in Malpighian tubules of *ecd*$^{1}$ larvae fed 20-hydroxyecdysone at the non-permissive temperature (see Figure 5).

The data would indicate that 20-hydroxyecdysone does not repress urate oxidase activity in tubules cultured *in vitro*. However, it is possible that the Malpighian tubules may have some sort of autonomous developmental clock and that they are competent to respond to the hormone only within a narrow "developmental window". It is possible that at 29$^{o}$C, when the larvae experience the peak urate oxidase activity this "window" is closed and the tubules are no longer competent to respond.

To test this hypothesis, Malpighian tubules were obtained from *ecd*$^{1}$ larvae raised at 29$^{o}$C at various times prior to peak urate oxidase activity. The tubules were then cultured *in vitro* in Schneider's medium with and without 20-

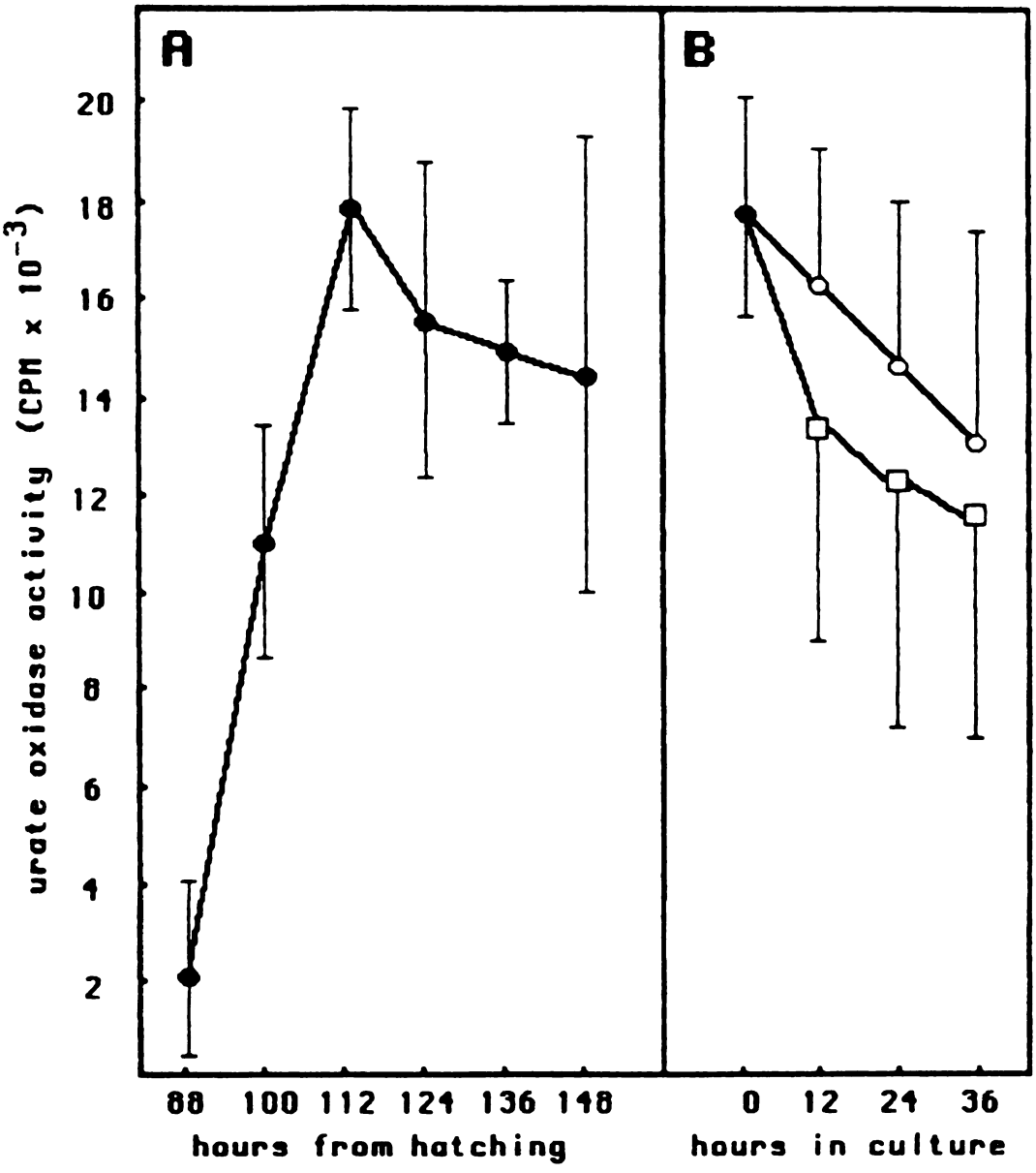


Figure 9. Urate oxidase activity of Malpighian tubules cultured in vitro at a time of maximum *in vivo* activity. Panel A: urate oxidase activity of *ecd*$^{1}$ third instar larvae reared on yeast at 29°C. Panel B: urate oxidase activity of Malpighian tubules cultured in Schneider's medium with (●) and without (○) $10^{-4}$M 20-hydroxyecdysone. The tubules were transferredto Schneider's medium from 112 hour old *ecd*$^{1}$ third instar larvae.

hydroxyecdysone and assayed for urate oxidase activity after 32 hours of culture. As shown in Figure 10, the urate oxidase activity of all *in vitro* cultured tubules declined slightly (but not significantly) during the 32 hours and there was no significant difference in activity between those tubules cultured in the presence of 20-hydroxyecdysone and those tubules cultured in the absence of 20-hydroxyecdysone. The decline in urate oxidase activity induced by 20-hydroxyecdysone *in vivo* (Figure 6) was not observed in Malpighian tubules of the same age cultured in vitro in the presence of the hormone (Figure 10).

These results indicate that 1) Malpighian tubules obtained from larvae during a time when urate oxidase activity is being induced will maintain the level of urate oxidase activity which the tubules exhibit at the time of transfer to Schneider's medium; 2) tubules transferred to synthetic culture medium at a time when urate oxidase activity is no longer being induced will show a decline in urate oxidase activity in culture at about the same rate as the decline in activity exhibited by larvae in the absence of endogenous 20-hydroxyecdysone; and 3) 20- hydroxyecdysone does not cause a precipitous drop in urate oxidase activity in *in vitro* cultured Malpighian tubules.

One possible interpretation of the data is that the steroid hormone 20-hydroxyecdysone does not act on the Malpighian tubules directly to repress urate oxidase, but rather must act through some other cell type. This

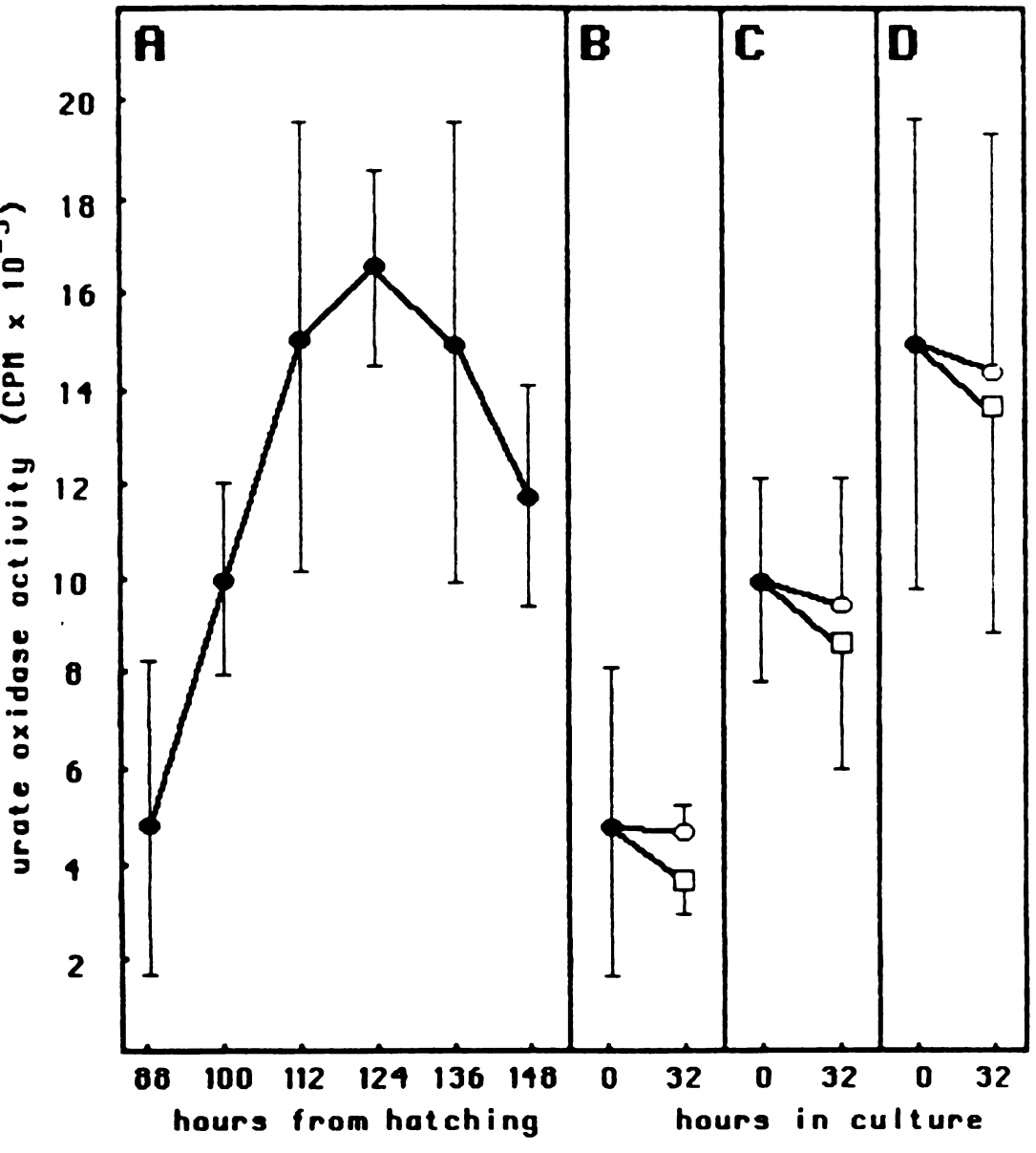


Figure 10. Urate oxidase activity of Malpighian tubules cultured in vitro in Schneider's medium with (■) and without (●) $10^{-4}$M 20-hydroxyecdysone. The tubules were transferred to Schneider's medium from 88 hour old *ecd*$^{1}$ third instar larvae (panel B); 100 hour old *ecd*$^{1}$ third instar larvae (panel C); and 112 hour old *ecd*$^{1}$ third instar larvae (panel D). Panel A: urate oxidase activity of *ecd*$^{1}$ third instar larvae, from which the tubules were obtained, reared on yeast at 29°C.

conclusion, however, may not be warranted in view of the following observations.

Third instar *ecd*$^{1}$ larvae reared at 29°C on yeast food were transferred to cellulose powder with and without 20-hydroxyecdysone for 24 hours at 29°C. At 12 hours and again at 24 hours, larvae from both treatment groups were assayed for urate oxidase activity. As the results presented in Figure 11 clearly show, the drop in urate oxidase activity of starving larvae is not in any way effected by the steroid hormone. That is, when *ecd*$^{1}$ larvae of the same age are fed yeast and 20-hydroxyecdysone the urate oxidase activity drops precipitously to almost zero (Figure 5, panel C), whereas starving larvae exhibit a drop in urate oxidase activity that is somewhat more gradual regardless of whether 20-hydroxyecdysone is ingested at the same time (Figure 11).

**Conclusions**

Collectively, all of the genetic and biochemical data presented in this chapter support the the notion that 20-hydroxyecdysone is implicated in the regulatory process by which urate oxidase activity declines precipitously at the time of puparium formation in Drosophila. There is no direct experimental evidence to support the hypothesis that 20-hydroxyecdysone acts directly on the genome of the Malpighian tubules causing the repression of transcription of the urate oxidase gene.

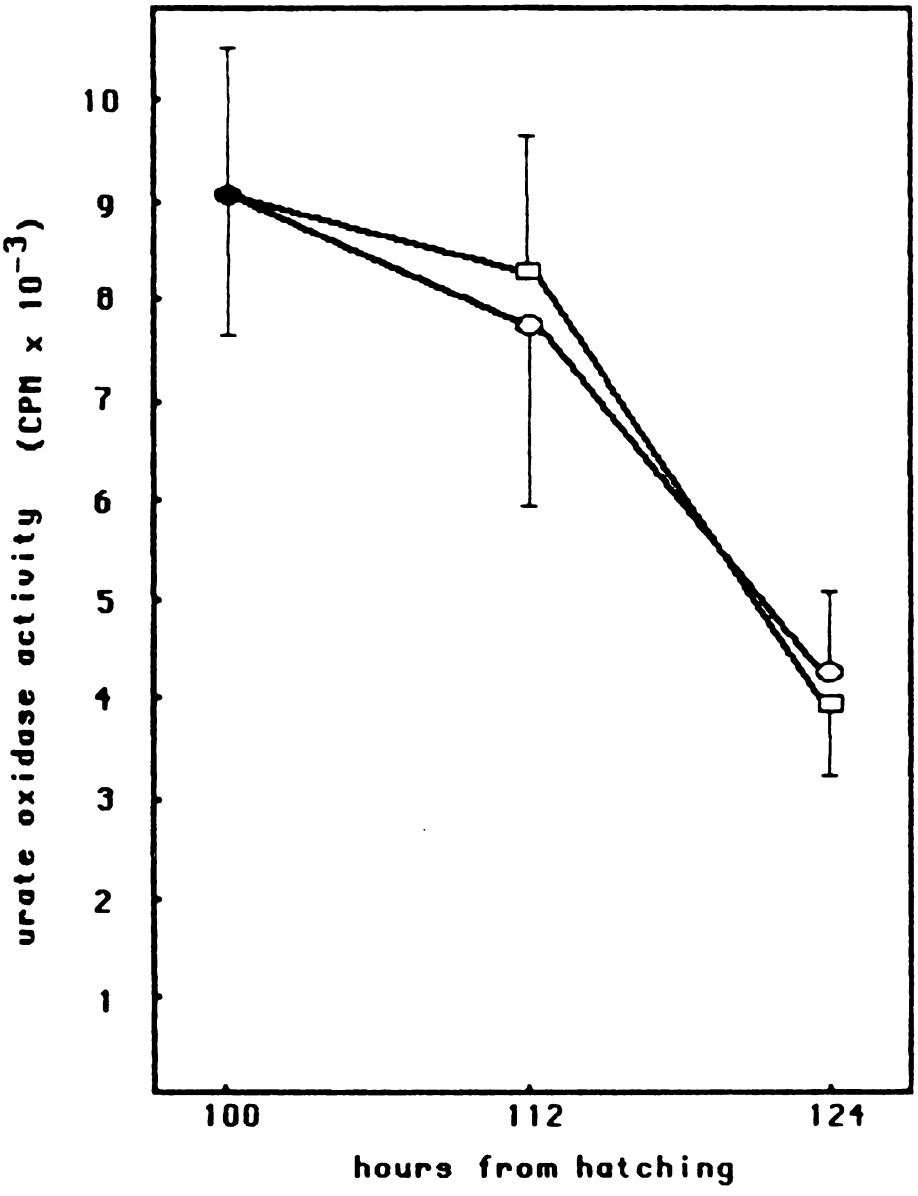


Figure 11. Urate oxidase activity of *ecd*[1] third instar larvae feeding on cellulose saturated with 0.8% sucrose (○) only and 0.8% sucrose with $10^{-4}$M 20-hydroxyecdysone (□). The larvae were transferred from yeast food at 100 hours and reared at 29°C for the duration of the experiment.

The hypothesis that 20-hydroxyecdysone directly represses transcription of the urate oxidase gene is not ruled out by the fact that a substantial drop in urate oxidase activity can not be initiated by 20-hydroxyecdysone in larval Malpighian tubules *in vitro*. As has been shown, the *in vivo* induced decline in urate oxidase activity is dependent on a specific physiological state of the larva. It may very well be that 20-hydroxyecdysone acts directly on the Malpighian tubules to repress the urate oxidase gene, but some other humoral agent absent in the starving larvae and also absent in Schneider's medium may be necessary for the synthesis and degradation of the urate oxidase peptide and mRNA.

## CHAPTER TWO

### Introduction

One goal of studying the urate oxidase gene will be to understand how the structure of the gene and its flanking sequencesinteracts with various effector molecules in the Malpighian tubules to modulate the gene's expression. To this end, it is imperative to identify all factors which act upon the tubules to induce and repress urate oxidase.

The data presented in the previous chapter support the hypothesis that urate oxidase activity is induced in both Drosophila larvae and adults by the same diffusible factor. A further correlation has been drawn between the induction of urate oxidase activity and food intake by the larvae. The same observation was reported by Friedman (1973) for adult wild-type Ore-R flies. Adult wild-type Drosophila raised on yeast have significantly higher urate oxidase activity than those starved for 24 hours.

This chapter will further explore the effect of diet on urate oxidase activity in the third instar Drosophila larva. The purpose of the experiments presented here is to obtain a better understanding of the physiological processes which control the induction of urate oxidase. This information may prove usefull in identifying the diffusible urate oxidase inducing factor.

**Effect of Diet on Urate Oxidase Activity**

Data presented in Table II of chapter one demonstrated that Malpighian tubules obtained from white prepupae are no longer competent to respond to the urate oxidase inducing factor. The decline of urate oxidase activity at the time of puparium formation has been shown to be due to the presence of 20-hydroxyecdysone. However, it has also been demonstrated that a weaker decline in urate oxidase activity can be initiated in the larvae by starvation.

The following experiment was performed to determine whether the decline of urate oxidase activity in starving larvae can be reversed by returning the larvae to food, or whether the activity is permanently repressed once a decline is initiated. Early third instar *ecd*$^{1}$ larvae raised on yeast food were shifted to 29$^{o}$C. When urate oxidase activity was at about half of the maximum possible activity, a group of the larvae were transferred to wet cellulose powder. After 12 hours of starvation, a group of the starved larvae was transferred to yeast food. Larvae were assayed for urate oxidase activity at 12 and 24 hours after the initial transfer to cellulose. As Figure 12 shows, urate oxidase activity declines during the initial 12 hours of starvation. But once the larvae are transferred to food, the urate oxidase activity is once again induced at the same rate as in the sister larvae which were never starved.

These results further confirm that the increasing level of urate oxidase activity in third instar larvae is directly

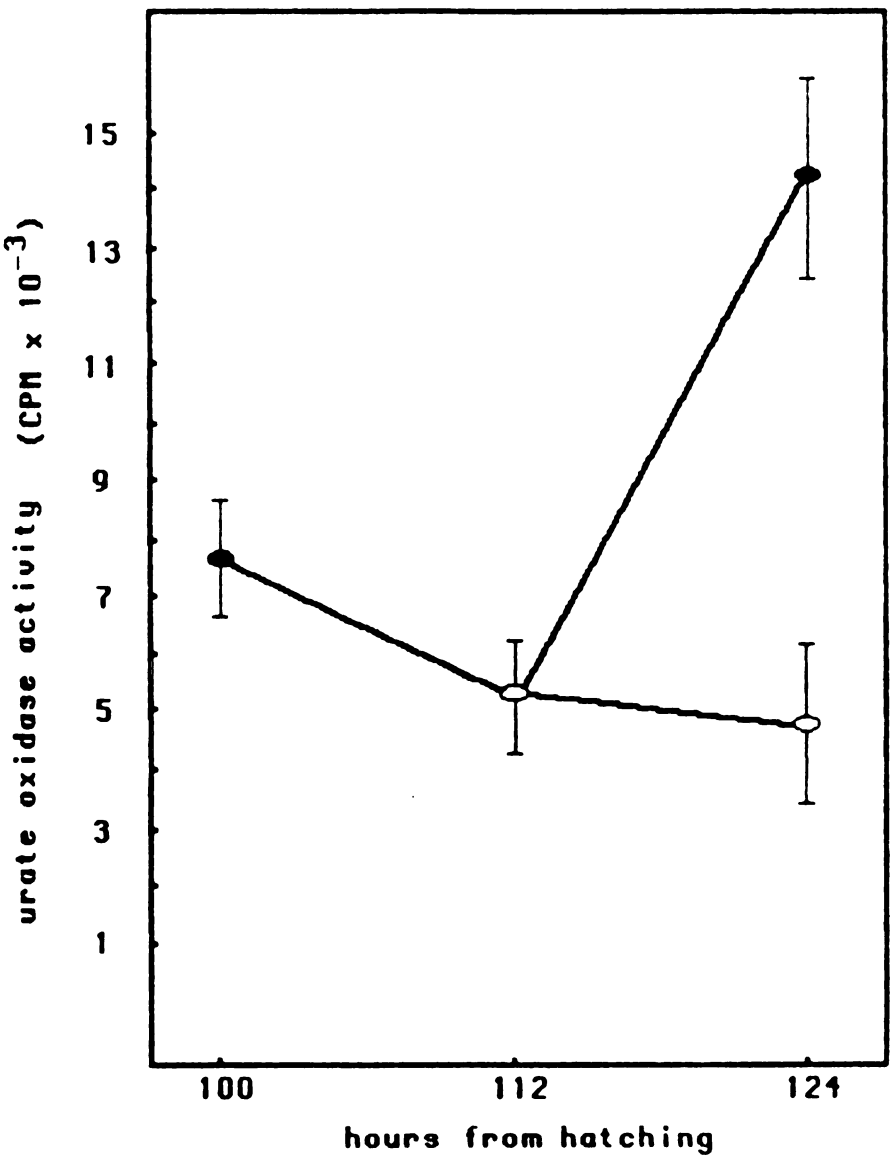


Figure 12. The effect on urate oxidase activity of feeding starved larve. At 100 hours of development, third instar ecd$^{1}$ larvae were transferred from yeast food to cellulose powder saturated with 0.8% sucrose (○). At 112 hours of development, a group of the starved larvea was transferred back to yeast food for 12 hours (●). The larvae were reared at 29°C for the duration of the experiment.

dependent on food intake. Presumably, the quantity of the diffusible urate oxidase inducing factor to which Malpighian tubules respond is modulated by the nutritional status of the larvae.

Drosophila, like many other insects and birds are uricotelic organisms in that they excrete nitrogenous wastes in the form of uric acid and/or its metabolites. Urate oxidase converts the waste product uric acid to allantoin. Allantoin is the major excretion product in the butterfly Papilio xuthus (Tojo and Yushima, 1972). There is also some evidence that allantoin may be the major excretion product in Drosophila when urate oxidase activity is present (Johnson et al., 1980a,b,c).

A number of experiments were designed to test which nitrogenous component of the larval diet is important for urate oxidase induction. In all of the previous experiments, larvae were reared on live yeast. In order to determine if live yeast was necessary for the induction of urate oxidase, ecd[1] larvae were raised on cellulose powder saturated with a solution of yeast extract. As the results in Table IV show, the induction of urate oxidase, which is inhibited by rearing the larvae on moist cellulose powder alone, proceeds normally on the yeast extract diet.

To determine if an increase in the nitrogen content of the diet will cause a greater induction of urate oxidase activity, yeast extract was supplemented with 0.4% yeast RNA (Sigma). This amount of RNA was chosen because it was found

Table IV. Urate oxidase activity of *ecd*[1] early third instar larvae* reared on wet cellulose and supplements at 29°C for 24 hours.

| Supplements | Urate oxidase activity (CPM) |
|---|---|
| 0.8% sucrose | 4,257 ± 530 |
| 6.0% yeast extract<br>0.8% sucrose | 24,726 ± 5,410 |
| 6.0% yeast extract<br>0.8% sucrose<br>0.4% RNA | 29,163 ± 3,582 |

*initial urate oxidase activity at 100 hours = 11,847 ± 538
The number of independent determinations = 4.

to be optimum in a defined diet (Burnet and Sang, 1963). A group of larvae was raised on yeast extract and another group was raised on yeast extract with 0.4% RNA. Both groups of larvae were assayed for urate oxidase activity after 24 hours. As data in Table IV shows, urate oxidase activity is induced on both diets and there is no statistically significant difference in activity on the RNA supplemented yeast diet as compared to the yeast diet without added RNA.

RNA is a component of yeast and yeast extract. It may be that the amount of dietary nitrogen present in yeast is enough to cause the maximum possible rate of urate oxidase induction. To explore this possibility, it was necessary to prepare a diet which was defined with respect to nitrogen containing compounds. The major source of dietary nitrogen is probably amino nitrogen in the form of amino acids and nitrogen from purine and pyrimidine compounds.

Early third instar ecd[1] larvae were divided into three groups. One group was reared on moist cellulose, the second on cellulose saturated with 5% casein, and the third on cellulose-casein supplemented with 0.4% RNA. After 12 hours at 29°C, the larvae were assayed for urate oxidase. As the results in Table V indicate, the casein diet alone is capableof inducing urate oxidase activity at about the same rate as a live yeast diet (Figure 3). There is no significant increase in urate oxidase activity when the casein diet is supplemented with RNA.

Table V. Urate oxidase activity of *ecd*[1] early third instar larvae* reared on wet cellulose and supplements at 29°C for 12 hours.

| Supplements | Urate oxidase activity (CPM) |
|---|---|
| 0.8% sucrose | 3,878 ± 822 |
| 6.0% casein<br>0.8% sucrose | 10,725 ± 1,498 |
| 6.0% casein<br>0.8% sucrose<br>0.4% RNA | 12,114 ± 1,527 |

* initial urate oxidase activity at 88 hours = 2696 ± 331
The number of independent determunations = 4.

Based on these results it would appear that amino nitrogen is reponsible for urate oxidase induction. However, since urate oxidase was maximally induced, any possible contribution by nucleic acid nitrogen would have been masked even if nucleic acid nitrogen can induce urate oxidase. To control for this possibility, early third instar *ecd*[1] larvae were reared on a number of diets of reduced (1.5% casein) amino nitrogen each supplemented with a different concentration of purine or nucleic acid. The larvae were assayed for urate oxidase activity after 12 hours at 29°C. As the results in Table VI show, low levels of casein (1.5%) induce urate oxidase at a slower rate than high levels of dietary casein (6.0%) (Table V). Supplementing the protein diet with other nitrogenous compounds at levels greater than that of casein did cause a slight increase in activity, but this increase was neither significant nor reproducible.

Even if the apparent increase in urate oxidase activity on a low protein, high nucleic acid diet were significant, the level of induction of urate oxidase is minimal in comparison to that observed in larvae reared on a high protein diet. Therefore, the most likely explanation for the dietary dependence of urate oxidase activity is that the waste nitrogen excreted by Drosophila as uric acid and allantoin is primarily the excess amino nitrogen from amino acids.

Table VI. Urate oxidase activity of *ecd*[1] early third instar larvae raised on low casein supplemented with purines and nucleic acids at 29°C.

| Diet | Urate oxidase activity (CPM) |
|---|---|
| 1.5% casein<br>0.8% sucrose<br>0.06% uridine | 4,182 ± 1,623 |
| 0.8% sucrose<br>0.06% uridine | 2,699 ± 345 |
| 1.5% casein<br>0.8% sucrose<br>0.06% uridine<br>0.2% RNA | 5,828 ± 2,052 |
| 1.5% casein<br>0.8% sucrose<br>0.06% uridine<br>2.0% RNA | 5,019 ± 684 |
| 1.5% casein<br>0.8% sucrose<br>0.06% uridine<br>0.2% hypoxanthine | 5,685 ± 1,110 |
| 1.5% casein<br>0.8% sucrose<br>0.06% uridine<br>2.0% hypoxanthine | 6,539 ± 1,534 |
| 1.5% casein<br>0.8% sucrose<br>0.06% uridine<br>0.2% inosine | 5,637 ± 1,212 |
| 1.5% casein<br>0.8% sucrose<br>0.06% uridine<br>2.0% inosine | 6,076 ± 1,690 |

The number of independent determinations = 4.

**Role of Purine de nove Synthesis in Urate Oxidase Induction**

Any waste nitrogen which is to be excreted via the uricotelic pathway other than excess purines has to pass through the amino acid pool. The amino acids glycine, glutamine, aspartate, and serine (Figure 13) are then utilized in the de novo purine synthesis pathway which leads through the formation of inosine monophospahte to the formation of uric acid and allantoin, the excretory products.

To test the hypothesis that the induction of urate oxidase is somehow effected by the *de novo* synthesis of purines, early third instar *ecd*[1] larvae were raised at 29°C on moist cellulose supplemented with very low dietary protein and a high concentration of the amino acids involved in *de novo* purine synthesis. After 24 hours, urate oxidase activity was determined. As the data in Table VII show, urate oxidase activity of larvae which ingested the precursors of purine *de novo* synthesis (Figure 13) was almost twice the activity of the larvae which were fed casein alone. Interestingly, feeding the larvae the precursors alone without additional casein failed to induce urate oxidase activity.

As indicated in Figure 13, there is one step in the de novo synthesis of purines which can be blocked by the glutamine analogs azaserine and 6-diazo-5-oxo-L-norleucine (DON). The effects of inhibition of purine biosynthesis by these compounds on urate oxidase activity was determined.

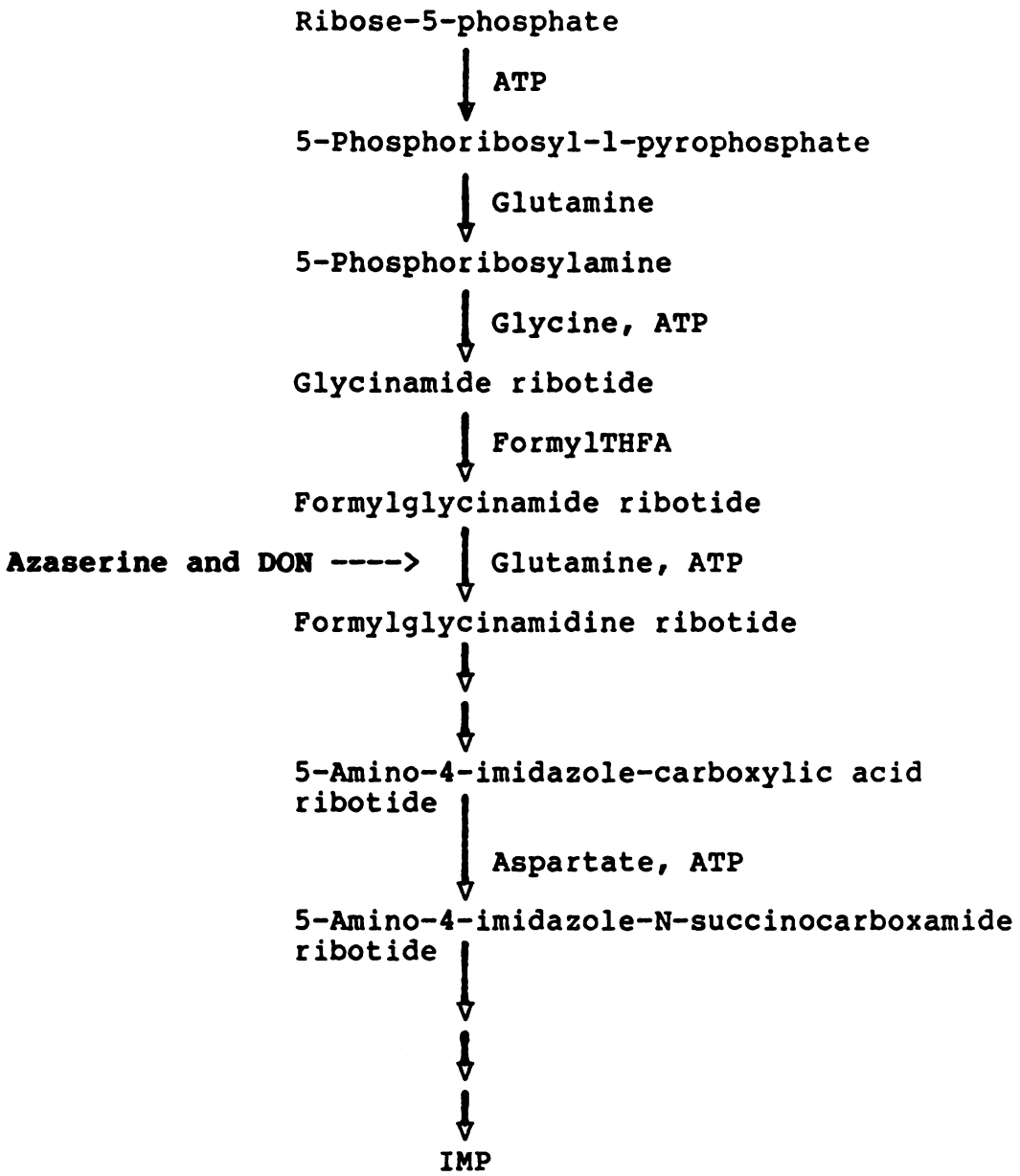


Figure 13. Azaserine and DON sensetive site in de novo purine biosynthesis. (Pittillo and Hunt, 1967). This site has been determined in bacteria and mammalian cells. Whether the same enzymatic step is inhibited by these antibiotics in Drosophila has not been confirmed.

Table VII. Urate oxidase activity of *ecd*[1] early third instar larvae raised on low casein supplemented with precursors of de novo purine synthesis at 29°C.

| Diet | | Urate oxidase activity (CPM) |
|---|---|---|
| 0.5% | casein | |
| 0.8% | sucrose | |
| 0.2% | glycine | |
| 0.2% | glutamine | 4,227 ± 957 (n=8) |
| 0.2% | aspartate | |
| 0.2% | serine | |
| 0.0003% | folate | |
| 0.057% | uridine | |
| 0.5% | casein | |
| 0.8% | sucrose | 2,488 ± 493 (n=8) |
| 0.057% | uridine | |
| 0.8% | sucrose | |
| 0.2% | glycine | |
| 0.2% | glutamine | |
| 0.2% | aspartate | 2,355 ± 531 (n=4) |
| 0.2% | serine | |
| 0.0003% | folate | |
| 0.057% | uridine | |

n = number of independent determinations.

Initially, early third instar *ecd*[1] larvae were transferred to cellulose soaked with a solution of 5% casein supplemented with different concentrations of azaserine. Urate oxidase activity was determined after 24 hours at 29°C. Azaserine at a concentration of $10^{-2}$M in the diet proved effective in inhibiting a further casein induced urate oxidase increase (Table VIII). Azaserine at $10^{-4}$M and $10^{-6}$M had no effect on urate oxidase induction (Table VIII).

Data in Table IX show the effect of $10^{-2}$M azaserine on urate oxidase activity at 12 and at 24 hours after feeding as a supplement in a 5% casein diet. The inhibition of urate oxidase activity is not complete at this concentration of azaserine. The activity more than doubled in the presence of the inhibitor. However, the induction of activity in the presence of azaserine may be due to physiological factors influencing the levels of the urate oxidase inducing factor which are independent of the inhibitory effects of azaserine. This interpretation is supported by the observation that very little if any increase in urate oxidase activity occurs in the presence of azaserine between 12 and 24 hours of the experiment (Table IX).

The effects of $10^{-2}$M DON on urate oxidase activity at 12 and at 24 hours after feeding as a supplement in a 5% casein diet are shown in Table X. The inhibitory effect of DON is very much like that of azaserine. That there is no increase in activity after 12 hours on the DON supplemented

Table VIII. The effects of varying concentrations of azaserine on urate oxidase activity induced by dietary casein.

| azaserine in diet | urate oxidase activity (CPM) |
|---|---|
| none | 9,649 ± 1,410 |
| $10^{-2}$M | 5,620 ± 1,048 |
| $10^{-4}$M | 9,790 ± 1,745 |
| $10^{-6}$M | 10,728 ± 1,759 |

Early third instar *ecd*[1] larvae were rerared on wet cellulose supplemented with casein and azaserine as indicated at 29°C for 24 hours.
Number of independent determinations = 6.

Table IX. Inhibition of casein induced urate oxidase activity by $10^{-2}$M azaserine at 12 and 24 hours of feeding at 29°C.

| diet | urate oxidase activity (CPM) 12 hours | 24 hours |
|---|---|---|
| 5.0% casein<br>0.8% sucrose | 3,763 ± 1,106 | 9,144 ± 1,566 |
| 5.0% casein<br>0.8% sucrose<br>0.2% azaserine | 3,139 ± 499 | 3,481 ± 1,146 |

Urate oxidase activity of early third instar *ecd*[1] larvae prior to transfer to casein diet was 1,660 ± 256.
Number of independent determinations = 6.

Table X. Inhibition of casein induced urate oxidase activity by $10^{-2}$M DON at 12 and 24 hours of feeding at 29°C.

| diet | urate oxidase activity (CPM) 12 hours | 24 hours |
|---|---|---|
| 5.0% casein 0.8% sucrose | 13,846 ± 2,506 | 16,456 ± 1,673 |
| 5.0% casein 0.8% sucrose 0.2% DON | 5,510 ± 1,290 | 8,240 ± 969 |

Urate oxidase activity of early third instar *ecd*[1] larvae prior to transfer to casein diet was 5,577 ± 2,106.
Number of independent determinations = 6.

diet is probably due to the fact that this experiment was initiated with larvae somewhat older than those used in the experiment with azaserine as the inhibitor (Table IX). In the experiment where azaserine was used as the inhibitor the initial activity of urate oxidase was 1660 units whereas in the one where DON was tested, the initial activity was 5510 units. When early third instar larvae are starved at a time when urate oxidase activity is very low (2500 units) the activity increases slightly during the first 12 hours of starvation (Figure 4, arrow b). But when older larvae which have high activity are starved, urate oxidase activity drops during the first 12 hours of starvation (Figure 4, arrow a). An explanation for this observation may be that since there is no urate oxidase activity present in the second instar, the excretion of waste nitrogen may be below optimum. This may cause an increase in the nitrogen pool which may, in turn, cause an increase in the amount of the urate oxidase inducing factor.

Between 12 hours and 24 hours of feeding on a DON supplemented diet, the larvae show a slight increase in activity (Table X). This may be due to metabolic degradation of some of the DON.

Regardless of any minor differences that may exist, urate oxidase activity induction by dietary protein is inhibited by both azaserine and DON. In both cases only the increase of activity is prevented at the maximum concentration of inhibitors tested. These results suggest

that urate oxidase is induced in the Malpighian tubules by some metabolite made as a result of de novo purine synthesis or that the level of the diffusible urate oxidase inducer is modulated in response to the activity or pool size of metabolites of the de novo purine synthesis pathway.

Various nucleotides, nucleosides, and purines can be ingested by Drosophila larvae and utilized in the formation of all the catabolites of inosine monophospahte (Figure 14), the end product of de novo purine synthesis (Johnson et al, 1980). To test the hypothesis that any excess purine nitrogen ingested by larvae will also induce urate oxidase activity, larvae were fed a casein diet supplemented with various concentrations of RNA, and azaserine or DON. The results of feeding larvae 2% RNA at the same time de novo synthesis is inhibited by azaserine are shown in Table XI. Although the larvae fed RNA have a slightly higher level of activity than the larvae fed casein and azaserine only, the increase is not significant and certainly is substantially less than the increase observed in larvae fed casein only without the inhibitor.

Results in Table XI also show that the effect of azaserine on a yeast extract diet is not significantly different from its effect on a casein diet. In both cases the increase of urate oxidase activity is inhibited.

The effect of adding various concentrations of RNA to the diet of casein and the inhibitor DON is shown in Table XII. Early third instar <u>ecd</u>$^{1}$ larvae were transferred to

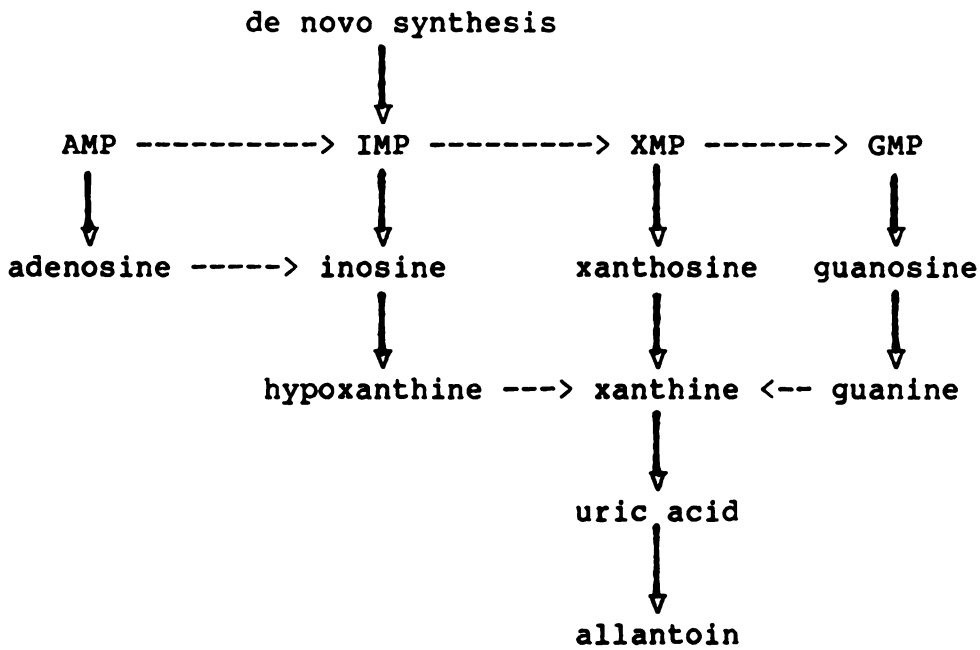


Figure 14. Catabolism of purines. AMP, adenosine-5'-monophosphate; IMP, inosine-5'-monophosphate; XMP, xanthosine-5'-monophosphate; GMP, guanosine-5'-monophosphate. (Johnson et. al., 1980a,b,c)

Table XI. The effect of dietary RNA on urate oxidase activity in larvae reared on casein and azaserine and the effect of azaserine on a yeast extract diet.

| diet | urate oxidase activity (CPM) |
|---|---|
| 5.0% casein<br>0.8% sucrose | 10,850 ± 2,976 |
| 5.0% casein<br>0.8% sucrose<br>0.2% azaserine | 4,862 ± 1,393 |
| 5.0% casein<br>0.8% sucrose<br>0.2% azaserine<br>2.0% RNA | 6,591 ± 1,675 |
| 5.0% yeast extract<br>0.8% sucrose | 12,312 ± 1,570 |
| 5.0% yeast extract<br>0.8% sucrose<br>0.2% azaserine | 5,993 ± 1,886 |

Early third instar *ecd*[1] larvae were transfered to wet cellulose and supplements as indicated and reared at 29°C for 24 hours.
Number of independent determinations = 6.

Table XII. Effect of dietary RNA and DON on casein induced urate oxidase.

| diet | inhibitor | urate oxidase activity (CPM) 12 hours | 24 hours |
|---|---|---|---|
| 5.0% casein<br>0.8% sucrose | DON<br>none | 5,510 ± 1,290<br>13,846 ± 2,506 | 8,240 ± 969<br>16,456 ± 1,673 |
| 5.0% casein<br>0.8% sucrose<br>1.0% RNA | DON<br>none | 5,950 ± 1,359<br>11,378 ± 5,445 | 7,459 ± 1,940<br>17,819 ± 6,665 |
| 5.0% casein<br>0.8% sucrose<br>3.0% RNA | DON<br>none | 7,192 ± 2,527<br>10,692 ± 3,644 | 8,589 ± 1,320<br>16,953 ± 7,378 |
| 5.0% casein<br>0.8% sucrose<br>5.0% RNA | DON<br>none | 5,902 ± 1,075<br>13,079 ± 2,980 | 6,685 ± 1,523<br>18,360 ± 3,705 |

Urate oxidase activity of early third instar *ecd*[1] larvae prior to transfer to casein diet was 5,577 ± 2,106. The inhibitor DON, when present, was at a concentration of $10^{-2}$M. Larvae were reared at 29°C on diets indicated.
Number of independent determinations = 6.

cellulose saturated with 5% casein, and 0%, 1%, 3%, or 5% RNA. A group of larvae was fed each of these diets with and without DON. Urate oxidase activity was determined at 12 and 24 hours following initiation of the experiment. The results indicate that DON effectively blocks further induction of urate oxidase. The RNA supplements do not overcome the inhibitory effectsof DON. In all treatment groups, regardless of the presence or absence of DON, the larvae fed RNA do not have significantly higher urate oxidase activity than the larvae fed casein alone. There is also no significant difference in activity among the groups of larvae reared on different amounts of RNA. Furthermore, whatever small differences in urate activity exist between these groups, there is no consistent correlation of urate oxidase activity and the amounts of RNA in the diet.

One major problem with using pharmacological inhibitors of any kind in a whole organism is the possible effect on more than one system or reaction. Consequently, it is difficult to interpret the results obtained with certainty. It would be preferable to have organisms such as *ecd*[1] which are mutant in one defined function so that the effects of that one physiological defect can be monitored. For this reason a Drosophila mutant was sought which disrupts the *de novo* synthesis of purines at a similar point in the pathway as azaserine or DON.

A search of the literature showed that no such mutant of the *de novo* purine synthesis pathway was known in

Drosophila. However, there were several mutants which seemed to be auxotrophic for purine and, therefore, required a purine source in their diet for growth as well as survival (Falk and Nash, 1974). Dr. D. Nash (University of Alberta, Alberta, Canada) graciously sent the mutant pur1-1 as well as the wild-type stock Amherst Ore-R from which the mutant was isolated.

To determine if this mutant has any effect on urate oxidase activity, both pur1-1 and Amherst Ore-R were raised on a yeast diet at 20°C. Urate oxidase activity was determined throughout the third instar larval stage. There was no difference observed in the behavior or development of these two strains although the pur1-1 mutant did take longer to reach puparium formation. As the data in Figure 15 indicate, there is no significant difference in the urate oxidase activity profile between the two strains.

It is doubtfull that the defect in the pur1-1 mutant is in the de novo purine synthesis pathway. The mutant pur1-1 will undergo normal development only if guanosine is supplied to the medium. Therefore, the defect in pur1-1 is probably in the conversion from inosine monophospahate (IMP) to guanosine monophospahte (GMP). The reason for believing this to be the case is that the feeding of any radioactively labeled purines such as adenosine, inosine or hypoxanthine to Drosophila larvae causes all purine monophosphates (AMP, IMP, and GMP) to be labeled in the same proportions (Johnson et al, 1980a,b,c). This also applies if [$^{14}$C] formate is

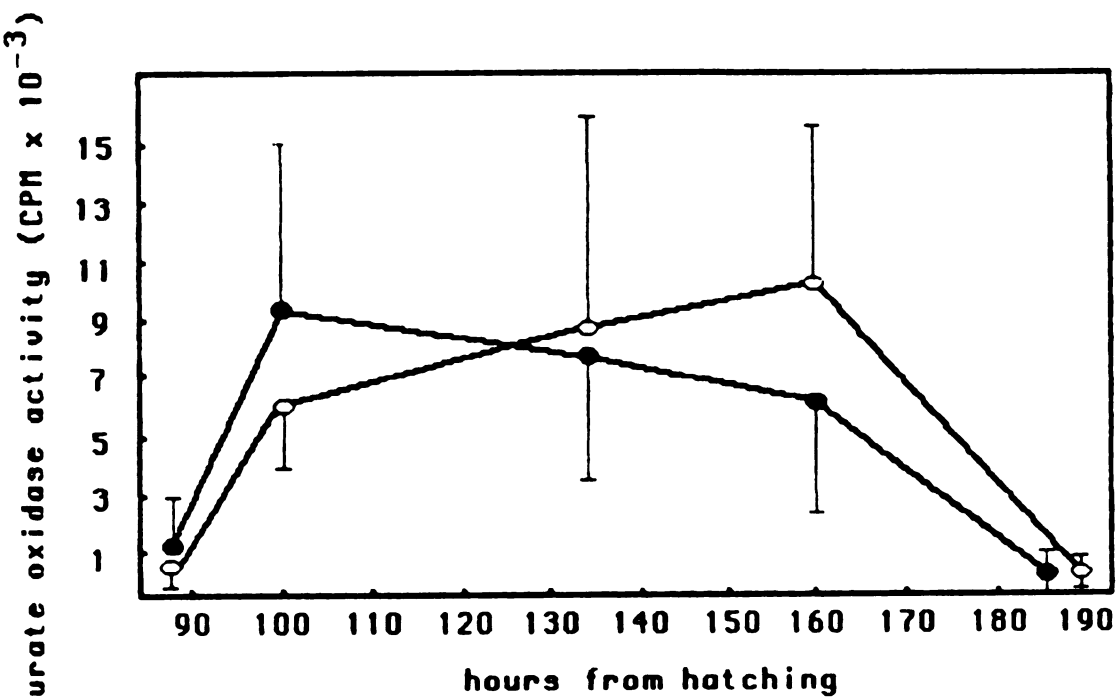


Figure 15. Urate oxidase activity of <u>purl-1</u> (Ø) and Amherst wild-type (●) third instar larvae reared on yeast at 20°C.

fed to larvae since all of the purines synthesized de novo are distributed to the three purine monophosphates in the same ratio (Johnson et al, 1980b). However, when [$^{14}$C] guanosine is fed to the larvae, most of it is converted to guanosine nucleotides. Very little is converted to other purines. Therefore, guanosine does not seem to be a good candidate for a purine molecule to be salvaged to restore the nucleotide pool.

It would appear then that the de novo purine synthesis pathway is probably intact in the *purl-1* mutant. Moreover, the *purl-1* mutation has no obvious effect on the quantity of urate oxidase activity during the third larval instar.

**Conclusions**

Data presented in this chapter have demonstrated a correlation between food intake and urate oxidase activity. That an increase in urate oxidae activity is correlated with high protein intake suggests that, in uricotelic insects, the main source of waste nitrogen is amino nitrogen from amino acids. However, it would appear that the molecule which acts as the inducer of urate oxidase or which is monitored by whatever system is responsible for urate oxidase induction, is probably a nitrogenous compound made by *de novo* purine synthesis because inhibition of purine synthesis by azaserine or DON inhibits urate oxidase induction even when larvae are fed a high protein diet.

Variations in protein content of diet has been shown to

affect catabolism of waste nitrogen in insects. In particular, xanthine dehydrogenase (XDH) activity is induced in both Drosophila (Collins et al., 1970) and the blowfly Aldrichina grahami (Higashino et al., 1977; Huynh et al., 1979). In the cockroach, increased protein concentration in diet causes an increased accumulation of uric acid in the fat body (Mullins and Cochran, 1975a,b) which is due to an increased rate of uric acid synthesis from [$^{14}$C]formate, [$^{14}$C]glycine and [$^{14}$C]xanthine (Engebretson and Mullins, 1983). Also, feeding a protein diet which is deficient in essential amino acids to the silkworm Bombyx mori results in increased secretion of uric acid (Horie and Inokuchi, 1975; Horie and Watanabe, 1983) since, presumably, the insect has to ingesta greater amount of food in order tosatisfy its need for essential amino acids.

Furhermore, in the blowfly, XDH activity is increased if the insect is reared on diets supplemented with precursors of de novo purine synthesis such as glycine and glutamine and also on diets supplemented with purines such as hypoxanthine and inosine (Huynh et al., 1979). When endogenous purines were analyzed, it was found that IMP was present at 40 to 80 fold greater levels on these diets as compared to diets without any supplements (Huynh et al., 1979).

No increase in urate oxidase activity was observed when Drosophila larvae were raised on food supplemented with purine compounds (Table VI). In this experiment, however,

the larvae were reared on the purine supplemented food for only 24 hours. In the experiment of Huynh and coworkers (1979) described above, the blowflies were reared on the purine supplemented diets for several days. It may be possible to induce urate oxidase activity in Drosophila by dietary intake of purine compounds if continuous ingestion takes place over a long enough period of time to cause an accumulation of endogenous purines. High endogenous concentrations of xanthine and hypoxanthine in the Drosophila mutant $\underline{ry}^2$ have been correlated with high urate oxidase activity (Friedman 1973).

It may also be possible to induce high urate oxidase activity in Drosophila by raising the flies on proteins low in essential amino acids.

By raising Drosophila under a variety of dietary conditions which cause induction of urate oxidase activity and then analyzing the endogenous levels of various purine compounds by HPLC, a purine compound may be identified the level of which may vary in correlation with urate oxidase activity.

## CHAPTER THREE

## Construction and Isolation of a Urate Oxidase cDNA Clone

### Identification of *in vitro* Translated Urate Oxidase

In order to isolate a cDNA for a particular mRNA, it is necessary to confirm the presence of the desired mRNA in the RNA preparation which will be used for cDNA construction.

The presence of urate oxidase mRNA was identified in a given RNA preparation by translating the RNA *in vitro* and immunoprecipitating the urate oxidase peptide. The immunoprecipitated *in vitro* translated products were then fractionated on SDS-polyacrylamide gels and visualized by fluorography.

When polyA$^+$ RNA from whole third instar larvae was translated *in vitro* in the presence of [$^3$H] leucine, only one dense band was observed in the fluorograph of the immunoprecipitated translation product (Figure 16, panel A). This band had a molecular weight of about 100,000 daltons. Drosophila urate oxidase is 41,000 daltons (Friedman and Barker, 1982). A 41,000-dalton peptide was not readily apparent although a faint band was visible just barely above background in some preparations.

When third instar larval polyA$^+$ RNA was translated *in vitro* in the presence of [$^{35}$S] methionine, a 41,000-dalton

peptide as well as a 100,00 dalton peptide was specifically immunoprecipitated from total larval polyA$^+$ RNA (Figure 16, panel B).

When polyA$^+$ RNA, obtained from Malpighian tubules of mid third instar larvae, was translated *in vitro* in the presence of [$^{35}$S] methionine, only one peptide band was specifically immunoprecipitated by anti-urate oxidase antibody (Figure 16, panel C). This peptide band has a molecular weight of 41,000 daltons as does purified Drosophila urate oxidase (Friedman and Barker, 1982).

Because no other peptide was specifically immunoprecipitated from the *in vitro* translated Malpighian tubule proteins, the 100,000 dalton peptide present in translates of whole larval polyA$^+$ RNA is probably not related to urate oxidase. Since the 41,000 dalton urate oxidase peptide is not apparent in the [$^3$H]leucine translates, urate oxidase has very few if any leucines.

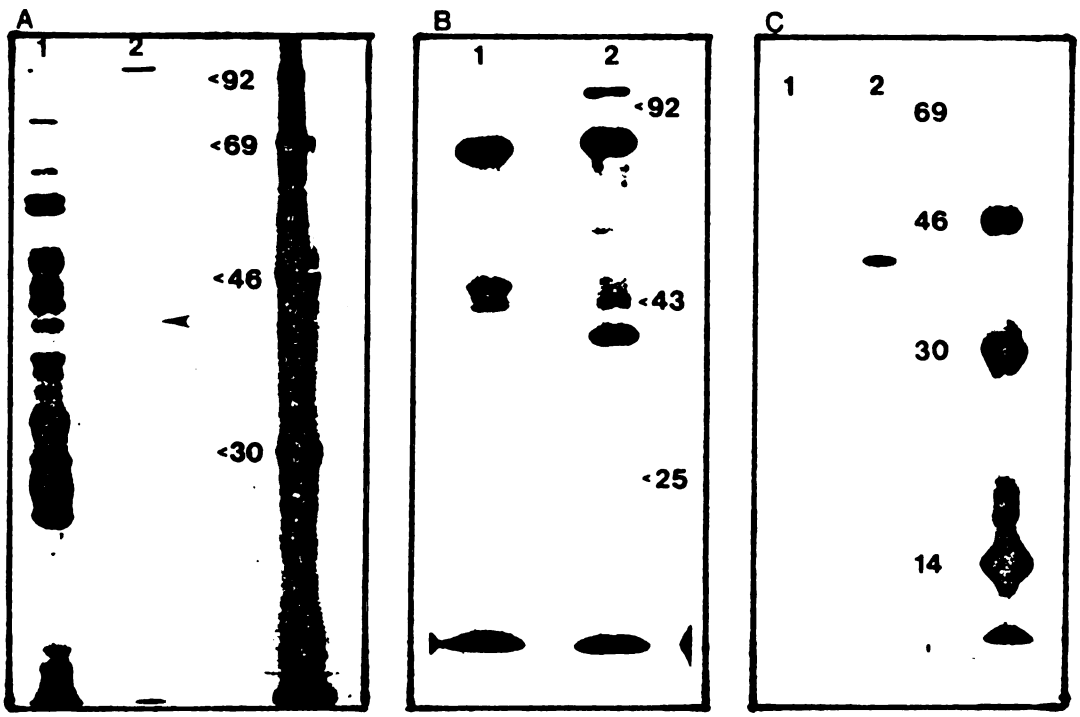


Figure 16. Fluorographs of dried SDS-polyacrylamide gels separating in vitro translated peptides synthesized in rabbit reticulocyte lysate systems. Panel A: [$^{3}$H] leucine labeled proteins translated from third instar larval polyA$^{+}$ RNA; Panel B: [$^{35}$S] methionine labeled proteins translated from third instar larval polyA$^{+}$ RNA; Panel C: [$^{35}$S]-methionine labeled proteins translated from polyA$^{+}$ RNA isolated from 500 larval Malpighian tubules. Lanes 1 and 2 in all panels contain peptides immunoprecipitated with preimmune (control) serum and anti-urate oxidase IgG respectively. Arrow in panel A indicates the presence of a light band in lane 2 which has the same molecular weight as urate oxidase.

**Protocol for Preparation of Malpighian Tubule cDNA Library**

The approach chosen for obtaining a cloned urate oxidase coding nucleotide sequence was the isolation of a urate oxidase cDNA. Since a 2,000 fold purification is necessary to obtain a homogeneous urate oxidase peptide preparation from whole adult $\underline{ry}^2$ Drosophila (Friedman and Barker, 1982), it is obvious that the urate oxidase mRNA would be relatively rare in a total Drosophila polyA$^+$ RNA preparation. In order for the selection of a urate oxidase cDNA clone from a cDNA library to be practical, it was necessary obtain polyA$^+$ RNA enriched for urate oxidase mRNA.

Since urate oxidase activity is confined only to the Malpighian tubules, and the tubules represent about 1% to 5% of the tissue mass of a third instar larva, polyA$^+$ RNA from Malpighian tubules would be enriched for urate oxidase mRNA about 20 to 100 fold.

The highest level of urate oxidase activity is found in $\underline{ecd}^1$ third instar larvae. Carefully staged early third instar larvae were reared on yeast until urate oxidase activity was about one half of maximum activity. Malpighian tubules were hand dissected from them in groups of five to ten in Drosophila Ringer. These tubules were then transfered in 5 ul of Drosophila Ringer to an Eppendorf capsule cooled in a dry ice-ethanol bath. The tubules were frozen immediately on contact with the capsule. The capsules containing the frozen tubules were then stored at -80$^o$C.

In all, about 3,000 such Malpighian tubules were obtained. PolyA$^+$ RNA was then extracted from the Malpighian tubules. Part of the RNA was used to ascertain the enrichment of urate oxidase mRNA achieved by this procedure. About one to two micrograms of Malpighian tubule polyA$^+$ RNA was translated *in vitro* in the presence of [$^{35}$S] methionine. The translation products were then divided into two aliquots. One was treated with preimmune serum, the other with anti-urate oxidase antibody, and then the antigen-antibody complexes were removed by pansorbin. The proteins in both aliquots that were not precipitated by either serum were fractionated on SDS-polyacrylamide gels. Comparison of these peptides on the fluorographs revealed that the urate oxidase peptide made up about 1% to 5% of the peptides translated from Malpighian tubule mRNA (data not shown). Therefore, about one in every one hundred cDNA clones in a cDNA library constructed from this Malpighian tubule polyA$^+$ RNA preparation was expected to be complementary to urate oxidase mRNA.

The protocol for constructing the Malpighian tubule cDNA library and for screening of the library is summarized below and described in detail in the next section. About 6 ug of Malpighian tubule polyA$^+$ RNA was used as template for the synthesis of cDNA by AMV reverse transcriptase. The single stranded cDNA was used as a template for the synthesis of double stranded cDNA molecules. Free 3' and 5' ends were created on these molecules by S1 Nuclease.

Poly-dC tails were synthesized on the 3' ends of the double stranded cDNA molecules with terminal deoxynucleotidyl transferase and these were then annealed to Pst I cleaved, poly-dG tailed pBR322 plasmids. These recombinant plasmids were used to transform E. coli RR1 and the transformed cells were selected on tetracycline plates. 1,200 cDNA clones were individually picked and stored. These were then screened in groups of five for their ability to hybrid select mRNA which would *in vitro* translate into a 41,000 dalton peptide recognized by anti-urate oxidase antibody.

**Details of Malpighian Tubule cDNA Library Preparation**

**A. First Strand Synthesis**

About 6 micrograms of Malpighian tubule polyA$^+$ RNA, isolated from about 2,000 Malpighian tubules, was added to a silanized 1.5ml Eppendorf capsule with 50 uCi of [$^{32}$P] dCTP (Amersham, 410 Ci/mmol) and 10 ul of a solution containing 10 mM dATP, dGTP, dTTP and 5mM dCTP (PL Biochemicals) in 10 mM Tris-HCl at pH 7.5. The contents was then frozen at -80$^{o}$C and lyopholized to dryness.

The following amounts of stock solutions were then added: 20 ul of 250 mM KCl; 20 ul of reverse transcription buffer containing 500 mM Tris-HCl pH 8.3, 50 mM $MgCl_2$, 50 mM DTT; 20 ul of Oligo dT12-18 solution (100 ug/ml); 20 ul of AMV reverse transcriptase (400 units); and 20 ul of water. All of these stock solutions were obtained from BRL,

Bethesda, Md. as part of a reverse transcriptase kit along with the AMV reverse transcriptase. The reaction mixture used was essentialy as recomended by BRL except that Actinomycin D (part of the kit) was not included in the reaction.

The reaction mixture was then incubated at 37°C for 60 minutes and terminated by immersion of the Eppendorf capsule into boiling water for 3 minutes (Wickens et al., 1978). The capsule was then cooled in ice for 5 minutes. This process also separates the cDNA strands from their RNA templates. Denatured protein was then pelleted by centrifugation in an Eppendorf centrifuge for 5 minutes at 4°C. The cDNA was then transferred to a new silanized 1.5 ml Eppendorf capsule. A 5 ul aliquot was removed for analysis (see section D).

**B. Second Strand Synthesis**

It is important that the first cDNA strand had been transferred to a new silanized capsule. Second strand synthesis will not proceed properly if the pelleted denatured material has not been removed.

Synthesis of the second strand was essentially as described by Wickens et al. (1978). The following stock solutions were added to the cDNA: 100 ul of second strand buffer containing 200 mM Hepes pH 6.9, and 90 mM KCl; 5 ul of a solution of 10 mM dATP, dGTP, dTTP and dCTP in water; and 100 units of E. coli Polymerase I. Deoxynucleotide triphosphates and E. coli Polymerase I were obtained from

PL Biochemicals, Milwaukee, Wis.; E. coli Polymerase I obtained from BRL or from New England Biochemicals did not yield clonable cDNA.

The reaction mixture was incubated at 15°C for 90 minutes at which time the reaction was terminated by the addition of 4 ul of 0.5 M EDTA. The reaction mixture was then extracted with 200 ul phenol and 200 ul of chloroform added sequentially with vortexing. After centrifugation the aqueous layer was transferred to a new silanized Eppendorf capsule. 10 ul of the reaction mixture was saved for analysis (see section D).

**C. S1 Nuclease Cleavage**

The synthesis of the second cDNA strand is primed by the 3' end of the first cDNA strand. This results in the formation of a single stranded hairpin loop connecting the two complementary cDNA strands. This single stranded loop is cleaved by S1 nuclease.

Since S1 nuclease is inhibited by deoxynucleotide triphosphates, most of these were removed by two ethanol precipitations in the presence of 2M ammonium acetate (Maniatis et al., 1982). The cDNA was then redissolved in 100 ul of water and the following solutions were added: 100 ul of S1 Buffer containing 100 mM NaAc pH 4.5, 400 mM NaCl, 2 mM $ZnSO_4$ and 1.0 % glycerol; and 10 units of S1 nuclease (BRL, Bethesda, Md.).

The reaction was incubated for 30 minutes at 37°C and

then terminated by the addition of 2 ul of 0.5 M EDTA and 200 ul of 0.1 M Trizma. The cDNA was extracted with 400 ul of phenol and 400 ul of chloroform added sequentially with vortexing and re-extracted with 400 ul of chloroform. The cDNA was then etahnol precipitated and redissolved in 100 ul of water. 5 ul of the reaction mixture was saved for anlysis (see section D).

**D. Analysis of cDNA Synthesis**

The synthesis of the first and second cDNA strands as well as the S1 nuclease cleavage was monitored. At the end of each of these procedures, a small aliquot of the cDNA was saved. These cDNA samples were then fractionated by electrophoresis in a denaturing alkaline agarose gel. The gel was then dried and autoradiographed. If everything worked correctly, the first strand should form a smear of DNA from 100 to about 1,500 basepairs; the double stranded cDNA before S1 nuclease cleavage should form a smear of single stranded DNA in the denaturing gel ranging from 200 to about 3,000 base pairs because the complementary DNA strands are connected; and the cDNA after S1 Nuclease cleavage should be of approximately the same size distribution as the first cDNA strand was.

The amount of S1 nuclease necessary to fully cleave all hairpin loops was determined empirically by digesting known concentrations of newly synthesized double stranded cDNA with various amounts of S1 nuclease. The products of these

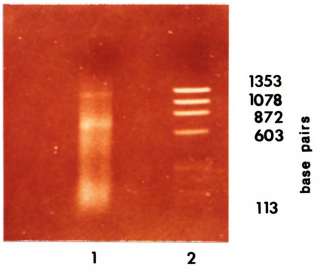


Figure 17. Size distribution of double stranded cDNA synthesized from whole Drosophila larval polyA$^+$ RNA. After S1 nuclease cleavage, approximately 5 ug of cDNA was fractionated by non-denaturing agarose gel electrophoresis. Lane 1: cDNA; Lane 2: DNA standards of size indicated.

digestions were monitored by electophoresis in denaturing alkaline agarose gels as outlined above. The quantity of S1 nuclease used in subsequent cDNA constructions was the lowest which completely converted the size distribution of all double stranded cDNA to that of the first strand.

The size of double stranded cDNA routinely produced by the above outlined procedure was also monitored by electrophoresis in non-denaturing agarose. All of the double stranded, S1 nuclease treated cDNA synthesized from about 6 ug of larval polyA$^+$ RNA was fractionated by non-denaturing agarose gel electrophoresis. The size distribution of the cDNA was visualized by ethidium bromide staining in comparison to known molecular weight standards. As shown in Figure 17, the double stranded cDNA after S1 Nuclease cleavage had a molecular weight distribution ranging from 100 base pairs to about 1,500 base pairs.

**E. Poly dC Tailing of cDNA**

In order to tail the cDNA with dCTP, it is necessary to have a preparation free of any contaminating deoxynucleotide triphosphates from previous reactions. This was achieved by two rounds of ethanol precipitation of the S1 nuclease treated cDNA in the presence of 2M ammonium acetate (Maniatis et al., 1982). This method was chosen over gel filtration because the recovery of the cDNA after ethanol precipitation is higher.

The Malpighian tubule cDNA was dissolved in 100 ul of

water. A 33 ul sample containing an estimated 1 ug of cDNA was added to a 1.5 ml silanized Eppendorf capsule. Also added were 10 ul of (5X) tailing buffer containing 500 mM potassium cacodylate pH 7.2, 10 mM $COCl_2$, and 1.0 mM DTT; and 5 ul of 10 mM dCTP.

The reaction mixture was prewarmed at 37°C for 10 minutes and 16 units of terminal deoxynucleotidyl transferase (BRL) was added in 2 ul of tailing buffer. The tailing reaction was incubated at 37°C for exactly 3 minutes and then terminated by quickly transferring the reaction tube to a 60°C waterbath for 2 minutes. Finally the tailed cDNA was stored on ice until used for annealing to dG-tailed pBR322.

Peacock and coworkers (1981) have shown that optimum transformation by hybrid plasmids is achieved when the poly-dC tails on the DNA insert have 15 dC residues and the poly-dG tails on the linearized pBR322 palsmid are about 20 dG residues long. The optimum range of tail lengths is very narrow in that a difference of as little as 5 nucleotides can decrease transformation efficiency by as much as 2 orders of magnitude.

The tailing reaction proceeds under conditions of enzyme excess and the variable which determines the length of the nucleotide tails is time of incubation (Peacock et al., 1981). Poly-dG tailed pBR322 plasmids were obtained from BRL. These had an average tail length of about 23 residues. The optimum length of poly-dC tails on the cDNA

was not determined directly. Instead, the time of the tailing reaction that produced poly-dC tailed cDNA inserts which gave the highest transformation efficiency after being annealed to the BRL poly-dG tailed pBR322, was determined. The optimum time of the dC tailing reaction was 3 minutes with the particular batch of chemicals and terminal transferase used.

**F. Annealing of dC-tailed cDNA to dG-tailed pBR322**

Approximately 80 ng of poly-dC tailed Malpighian tubule cDNA was combined with 200 ng of poly-dG tailed pBR322 in 400ul of annealing buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 150 mM NaCl). The annealing mixture was incubated at 58$^{o}$C for 90 minutes after which time it was stored on ice until used to transform E. coli RR1.

**G. Transformation of E. coli with Hybrid Plasmids**

Peacock et al. (1981) have shown that E. coli strain RR1 supports the highest transformation efficiency of hybrid plasmids constructed by annealing DNA molecules with complementary nucleotide tails. Hanahan (1983) optimized a procedure for transformation of E. coli cells with plasmids. Therefore, in the final steps of constructing the Malpighian tubule cDNA library, E. coli RR1 cells were transformed by the Hanahan transformation protocol.

About 1 ml of an overnight growth of E. coli RR1 was used to innoculate 200 ml of SOB medium (2% Bacto Tryptone;

0.5% Bacto Yeast Extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; and 10 mM $MgSO_4$). The density of the cells should be about 0.01 $A_{550}$ at time of innoculation. The cells were then grown at 37°C in an orbital air shaker at 275 revolutions/minute for 2 to 2.5 hours until the cell density reached 0.30 $A_{550}$. The flask was then cooled in ice water for 15 minutes.

30 ml of the cell supension were then transferred to sterile 50 ml Corning culture tubes and the cells were pelleted for 12 minutes at 3,000 RPM at 4°C. The medium was then decanted and each cell pellet was resuspended in 10 ml of TFB buffer (10 mM MES-KOH pH 6.3; 100 mM RbCl; 45 mM $MnCl_2$; 10 mM $CaCl_2$; and 3 mM $HACOCl_3$).

The cells were then incubated in the TFB buffer for 15 minutes at 4°C after which they were again pelleted at 3,000 RPM for 10 minutes at 4°C. The buffer was decanted and the cells in each tube were resuspended in 2.4 ml of TFB buffer. To each tube was then added: 84 ul of DMSO, after 5 minutes 100 ul of DTT (0.347gm/1 ml), and after 10 minutes another 84 ul of DMSO. After an additional 10 minutes, about 140 ng of recombinant plasmid was added to each of two tubes. The tubes were then incubated for 30 minutes. All of these operations were carried out in the cold room at 4°C.

At the end of the 30 minute incubation, the tubes were submerged in a 42°C waterbath for exactly 2.5 minutes. The time duration of this heat shock was determined empirically and needs to be optimized for the volume of cell suspension

involved as well as the size and commercial source of the plastic container. Immediately after the heat shock the tubes were submerged in ice water for 5 minutes after which time 10 ml of SOC medium (same as SOB medium above but with 1% glucose instead of the Mg salts) was added to each tube.

The tubes were incubated in an orbital air shaker at 225 revolutions/minute for 60 minutes at 37°C. 200 ul of the cell suspension was spread with a glass rod on each of 50 tetracycline supplemented (10 ug/ml tetracycline) LM agar plates (2% Bacto Tryptone; 0.5% Bacto Yeast; 10 mM NaCl; and 2.5 mM KCl). After the plates were incubated for 24 hours at 37°C, each of 1,200 5ml culture tubes containing 500 ul of freezing medium were innoculated with cells from one tetracycline resistant colony. These tubes were incubated over night at 37°C then stored frozen at -80°C. The remaining 20,000 or so colonies were scraped from the plates and resuspended in freezing medium. This Malpighian tubule cDNA library was also stored frozen at -80°C.

The poly-dC tailed cDNA was inserted into poly-dG tailed, Pst I cleaved pBR322. This recombinant plasmid carries a teracycline resistance gene. The RR1 E. coli strain is teracycline sensitive. All colonies of RR1 cells which formed on tetracycline supplemented plates should, therefore, contain recombinant cDNA plasmids.

Twenty randomly selected tetracycline resistant colonies were analyzed for the presence of plasmid, and plasmids were purified by small scale alkaline-SDS lysis

(Maniatis et al., 1982) from each colony. Furthermore, when the transformation protocol was carried out with the poly-dG tailed pBR322 only, virtually no tetracycline resistant colonies were recovered. Therefore, it was concluded that virtually all of the tetracycline resistant colonies recovered from the transformation with Malpighian tubule cDNA.pBR322 plasmids represent cDNA clones.

The size range of Malpighian tubule cDNA inserts cloned was determined by extracting plasmids from randomly picked colonies by small scale alkaline-SDS lysis, cleaving the plasmids with Pst I, and fractionating the products on non denaturing agarose gels. A representative gel is shown in Figure 18. The inserts range in size from about 100 base pairs to 1,100 base pairs with the average insert size being about 300 base pairs.

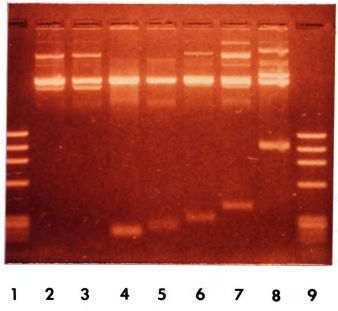


Figure 18. Agarose gel of Pst I cut Malpighian tubule cDNA-pBR322 plsmids. Of 1,200 individually isolated cDNA clones, 7 were selected at random. These were extracted by alkaline-SDS lysis, digested with Pst I, and the size of the excised cDNA fragments was determined on a 1.5% agarose gel. Lanes 2, 3, 4, 5, 6, 7, and 8 each contain the DNA of one of these plasmids. Lanes 1 and 9 contain DNA standards of size indicated. Lanes 2 and 3 each show a Pst I excised cDNA fragment of about 100 base pairs which is not visible in the photograph.

**Isolation of Urate Oxidase cDNA From the Malpighian Tubule cDNA Library**

As explained previously, the frequency of urate oxidase cDNA in the Malpighian tubule cDNA library was expected to be about one percent. This is a high enough frequency to make screening of the library by hybrid selection practical. Initially, 250 cDNA clones from the Malpighian tubule cDNA library were screened in groups of five. The cDNA clones in the group which gave a positive signal were then re-screened individually.

**Preparation of Plasmids**

For the initial screening, cDNA plasmids were prepared in groups of five. Each 100-ml of LB broth was innoculated with 100-ul of each of five overnight growths of Malpighian tubule cDNA clones. The cells were then grown at 37$^{o}$C in an orbital air shaker for about 2 hours at 280 RPM to 0.5 $A_{560}$. 75-ul of chlorenphenicol (34mg/ml in 100% ethanol) was then added to each growth and the cells were incubated for another 20 to 24 hours.

The cells from each group were then collected by centrifugation and resuspended in 2-ml STE buffer (0.1M NaCl, 10mM Tris-HCl pH 8.0, 0.1mM EDTA). 100ug of lysozyme (20mg/ml) was then added and the cells were incubated on ice for 20 minutes. The lysed cells were transferred to a silanized glass vial and the contents was brought to a boil. The glass vial was then immersed in boiling water for

exectly 0.6 minutes and immediately cooled on ice.

The clot was then transferred from the glass vial with the aid of a small spatula to an Oakridge tube and the denatured protein and chromosomal DNA was pelleted at 25,000 RPM for 30 minutes at 4°C in a Ti50 rotor. The supernatant was then extracted with an equal volume of phenol and chloroform, re-extracted with chloroform and ethanol precipitated.

The plasmids were then resuspended in 1-ml of water and incubated with 40-ul of DNAse free RNAse (10mg/ml) at 37°C for 2 hours. The plasmids were then extracted with phenol and chloroform twice, re-extracted with chloroform and ethanol precipitated. The high molecular weight DNA clot that formed was removed with forceps.

The plasmid pellet was dried and resuspended in 250-ul of water. This cDNA plasmid preparation was then stored frozen at -20°C.

**Binding of cDNA to Nitrocellulose Filters**

95-ul (approximately 5 to 10 ug) of each frozen cDNA preparation was transferred to a silanized Eppendorf capsule. The capsule was immeresed in boiling water for 10 minutes to denature the DNA. The capsule was then cooled on ice and 304.8-ul of denaturation buffer (5-ml formamide, 1.62-ml formaldehyde, 1.0-ml TE buffer) was added. The DNA was denatured at 55°C for 15 minutes and then cooled on ice.

The denatured DNA was then transferred to a test tube

containing 2-ml of 6x SSC. After mixing thoroughly, the DNA was suctioned onto 0.45 micron nitrocellulose (prewashed in 6x SSC at 68°C for 60 minutes) using a Schleicher and Schuell filter manifold. Each DNA preparation was applied to four filter wells. The circles of filter containing each DNA preparation were punched out using a baked paper-punch and collected in silanized Eppendorf capsules. The filters were then dried and baked at 80°C under vacuum for 2 hours.

At this point the filters were washed as follows. To each set of four filter disks containing a group of five cDNA clones in a single Eppendorf capsule, 500-ul of hybridization buffer (50% formamide, 10mM PIPES pH 6.4, 0.4M NaCl and 2% SDS) was added. The capsules were incubated for 2 hours at 65°C and the filters were then washed 5 times with 2-ml of water at room temperature. The filters were then dried under vacuum and stored at -20°C.

**Hybridization Selection of RNA**

Hybridization selection was carried out by the procedures of Parnes et al. (1981) and Gurney et al. (1982). For each set of filter bound cDNAs, 25-ul of hybridization solution (50% formamide, 10mM PIPES pH 6.4, 0.4M NaCl and 2% SDS) containing 50ug of third instar larval polyA$^+$ RNA was prepared. Before addition to the filters, the RNA was denatured at 65°C for 10 minutes. The RNA was then added to the capsules containing the filters and the capsules were

incubated at 50°C for 2 hours. At the end of the incubation 250-ul of Parnes buffer (10 mM Tris-HCl pH 7.6, 0.15 M NaCl, 1.0 mM EDTA) was added to the filters, mixed with the hybridization solution and the mixture was then removed from the capsule.

The filters were washed 5 times with 2-ml of Parnes buffer containing 0.5% SDS at 65°C and then 2 times with Parnes buffer (no SDS) at 65°C. The filters were then incubated for 20 minutes at 55°C in 200-ul of Gurney's modified Denhardt buffer (0.15M NaCl, 2.5 mM EDTA, 30 mM Tris-HCl pH 7.2, 25 mM $Na_3PO_4$, 0.22 mM Na pyrophosphate, 25ug/ml salmon sperm DNA, 1mg/ml Ficoll 400, 1mg/ml dextran sulphate, 1mg/ml BSA, and 1% SDS). The Gurney buffer was removed and the filters were washed twice with Parnes buffer (no SDS) and twice with TE buffer (20 mM Tris-HCl pH 7.0, 2.0 mM EDTA) at 55°C.

The specifically bound RNA was eluted from the filters by the addition of 300-ul of water containing 10-ug of calf tRNA and the capsule was immersed in boiling water for 90 seconds. The water was then quickly transferred to a new silanized capsule and the RNA was ethanol precipitated.

Urate oxidase polyA$^+$ RNA was then identified by *in vitro* translation, immunoprecipitation and SDS-polyacrylamide electrophoresis as described in the appendix. Data in Figure 19 shows the identification of a urate oxidase cDNA clone from the Malpighian tubule cDNA library.

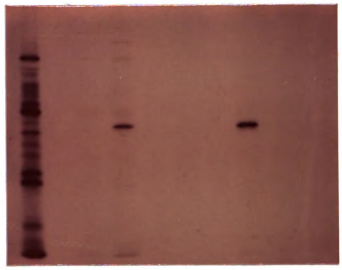


Figure 19. SDS-polyacrylamide gel electrophoresis of *in vitro* translated proteins from mRNA hybrid selected by Malpighian tubule cDNA. Lane 1: total labeled protein from the in vitro translation of total polyA$^+$ RNA used in the hybrid selection; Lane 2: protein immunoprecipitated from the translation reaction (lane 1) by preimmune serum; Lane 3: protein immunoprecipitated from the translation reaction (lane 1) by anti-urate oxidase serum; Lanes 4 - 8: protein immunoprecipitated by anti-urate oxidase serum from *in vitro* translations of RNA hybrid selected by individual Malpighian cDNA clones 61 - 65 respectively. Lanes 6 identified the recombinant plasmid (pcDNAU063) which contains a 520 base pair (data not shown) cDNA complementary to Drosophila urate oxidase mRNA.

## APPENDIX

### Materials and Methods

### Drosophila Strains

The wild-type strain Ore-R and the xanthine dehydrogenase deficient strains $ry^2$ and ma-1 were obtained from Dr. R. Woodruff of the National Drosophila Stock Center, Bowling Green, Ohio, and were maintained on cornmeal mollasses yeast food at 25°C (Friedman and Johnson, 1977). The temperature sensitive mutant $ecd^1$ was obtained from Dr. A. Garen, Yale University. At the non-permissive temperature of 29°C $ecd^1$ larvae fail to make or accumulate 20-hydroxyecdysone (Garen et al., 1977) and were maintained at the permissive temperature of 20°C on the same standard food. The purine requiring mutant pur1-1 and the wild-type strain Amhurst Ore-R were obtained from Dr. D. Nash, University of Alberta (Falk and Nash, 1974). These were maintained at 20°C and 25°C on the same standard food.

### Timing and Rearing of Larvae

Young $ecd^1$ adults were reared on a rich yeast-honey diet for three to five days. Eggs were then collected over a two hour period on paper spoons containing cream of wheat-mollasses food. After 32 hours at 20°C all larvae were

removed from the spoons and new larvae were allowed to hatch during a two hour period. These newly hatched larvae were then distributed to 90 X 50 mm glass crystalizing dishes (30 larvae per dish) containing cream of wheat-mollasses food and live yeast. The newly hatched larvae were maintained at 20°C for at least 88 hours (to early third instar) before being used for temperature-shift experiments.

For large scale isolation of timed larvae, eggs were collected on cream of wheat-mollasses food in large (1500-ml) jars over a six hour period. The eggs were then transferred to 2,000-ml and 4,000-ml erlenmeyer flasks containing cream of wheat-mollasses food and live yeast. The larvae were then harvested from the food by adding 20% sucrose solution to the flasks and allowing the larvae to float to the surface and accumulate in the narrow mouth. This method is less accurate then the one described previously, but much more practical when gram quantities of larvae of an approximate age (± 12 hours) must be obtained.

**Determination of Urate Oxidase Activity**

The activity of urate oxidase was measured either spectrophotometrically (Friedman, 1973) or by a radiochemical assay (Friedman and Merril, 1973). The radiochemical assay for urate oxidase activity measures the rate of formation of [$^{14}$C] allantoin from [$^{14}$C] uric acid and, as modified (Friedman and Johnson, 1977), can detect activity in a single set of Malpighian tubules from one

Drosophila. Malpighian tubules from Drosophila were obtained by submerging and dissecting the larva or adult in 0.5 M sucrose containing 1.7 mM EDTA, pH 6.9 at room temperature. The tubules were then transferred on the tip of a glass needle to a 1.5 ml Eppendorf capsule containing 15 ul of 11 mM boric acid, 1.4mM EDTA, and 0.18% Triton X-100 at pH 9.05. The tubules were incubated in this mixture at 4°C for 20 minutes, which disrupts the cells and releases urate oxidase into the assay mixture. At the end of this incubation period, 0.35 uCi of [2-$^{14}$C] uric acid (Amersham, 57 mCi/nmol) in 10 ul of water was added to initiate the reaction and the assay mixture was immediately incubated at 25°C. At two minutes and again at four minutes after the initiation of the reaction, 5-ul aliquots of the reaction mixture were removed and spotted 2 cm from the bottom of a MN-300 polyethyleneimine-impregnated cellulose thin-layer chromatography plate. Each spot was seperated by 2 cm. The [$^{14}$C] allantoin was then seperated from the [$^{14}$C] uric acid by ascending development in 0.15 M NaCl in 20% ethanol. The allantoin spot was identified as previously described (Friedman and Johnson, 1977; Friedman and Merril, 1973) and cut out of the plastic-backed cellulose plate. The amount of [$^{14}$C] allantoin formed was determined in a Beckman LS7500 liquid scintillation spectrometer. Urate oxidase activity is expressed as counts per minute of [$^{14}$C] allantoin formed from [$^{14}$C] uric acid per minute of reaction time per one set of Malpighian tubules.

The spectrophotometric assay for urate oxidase (Friedman, 1973) was carried out by adding 1 ul to 20 ul of homogenate to 750 ul of a solution containing 11 mM of boric acid and 1.4 mM EDTA at pH 9.05. The reaction was initiated with 60 ul of freshly prepared 0.04% uric acid. Urate oxidase activity is measured by monitoring the decrease of absorbance at 292 nm per minute of reaction time.

**Transplants of Malpighian Tubules**

Malpighian tubules were dissected from Ore-R and $ry^2$ newly emerged adults, $ecd^1$ larvae, and $ecd^1$ white pupae in Drosophila Ringer (0.128 M NaCl, 1.80 mM $CaCl_2$, 1.34 mM KCl, and 2.38 mM $NaHCO_3$) and transplanted into newly emerged Ore-R and $ry^2$ adult females as described (Friedman and Johnson, 1977). In experiments where Malpighian tubules were obtained from larvae or pupae only the anterior pair of tubules was transplanted. The posterior pair of tubules was assayed immediately to estimate urate oxidase activity of the donor anterior pair at the time of transplantation. The urate oxidase activity in the anterior pair is not significantly different from the posterior pair. The adult host carrying the transplanted anterior tubules were then placed into vials containing either the cornmeal-mollasses-yeast food supplemented with a yeast-honey mixture or into vials containing two Kimwipes soaked with water. The host tubules and the transplanted larval tubules were retrieved by submerging and dissecting the hosts in the sucrose-EDTA

solution described above. The donor larval tubules were distinguished from the host by two criteria: The *ecd*$^{1}$ Malpighian tubules are white, whereas the *Ore-R* and the *ry*$^{2}$ tubules are yellow. In addition the host tubules were attached to the alimentary canal whereas the donor larval tubules remained unattached to the alimentary canal. The latter criterion was used in the retrieval of cultured adult tubules since both host and adult donor tubules are yellow.

**SDS-Polyacrylamide Gel Electrophoresis**

Samples for electrophoresis were prepared by dissecting larvae or pupae in Drosophila Ringer at room temperature. Malpighian tubules were immediately transfered to a 1.5 ml Eppendorf capsule containing about 100 ul of Ringer at 4°C. When 10 to 15 tubules were collected they were pelleted for one minute in an Eppendorf table top centrifuge and the Ringer solution was removed. The tubules were then stored at -80°C. Just before electrophoresis, 20 to 40 ul of sample buffer (Laemmli, 1970) was added to the frozen tubules. The 1.5 ml capsule was then placed in boiling water for 10 minutes. A 10% polyacrylamide slab gel (1.5 mm or 0.75 mm thick, 100 mm long and 140 mm wide) was prepared according to Laemmli, 1970. Electrophoresis was carried out at 22°C at a constant current of 1 milliampere per 0.1 mm thickness of gel after which proteins in the gel were stained with Coomassie Brilliant Blue R or with silver according unpublished modifications by Dr. R. C. Switzer of

his original procedure (Switzer et al., 1979). The modified silver stain procedure results in a clear background while retaining high sensitivity.

**Immunoprecipitation of Urate Oxidase from Malpighian Tubules**

Malpighian tubules were obtained from ecd$^{1}$ larvae and stored in the same manner as those obtained for the SDS-PAGE (described above). About 60 tubules were disrupted in 10 ul of 1 mM borate (pH 9.0), 15.7 mM EDTA, and 1% NP-40 in a Kontes microhomogenizer at 4$^{o}$C. The homogenate was transfered to a 1.5 ml Eppendorf capsule and the homogenizer was washed with an additional 55 ul of the borate-EDTA-NP-40 buffer. The wash was added to the homogenate, the sample was centrifuged for 5 minutes at 4$^{o}$C in an Eppendorf centrifuge and 25 ul of the supernatant was then transfered to each of two 1.5 ml test tubes containing 44 ul of pre-immune rabbit IgG and 40 ul of rabbit anti-Drosophila urate oxidase IgG (Kral et al.,1982), respectively. After 2 hours of incubation at 4$^{o}$C, the Malpighian tubule protein-IgG mixtures were transferred to 1.5 ml test tubes each containing a 15 ul pellet of swolen protein A-Spharose and incubated at 4$^{o}$C for one hour with frequent agitation. The Malpighian tubule proteins that did not bind to antibodies were then separated from protein A-Sepharose bound IgG by filtration through glass wool. To each of the filtrates, a one third volume of four times concentrated Laemmli sample buffer was added and the samples were subjected to SDS-

polyacrylamide gel electrophoresis as described above.

**Immunoprecipitation of Urate Oxidase peptide from *in vitro* Translates**

Rabbit reticulocyte translates were diluted 1:1 with NP40 buffer (phosphate buffered saline, pH 7.5 containing 1% NP-40). The diluted translates were then mixed with 2 ul of preimmune serum (per 50 ul of translate) at 4°C for 1 to 12 hours. The mixture was then added to a 20 ul pellet of hydrated protein-A sepharose. The protein-A sepharose was then kept suspended for 30 to 60 minutes at 4°C. The protein-A sepharose-IgG-adsorbed peptide complexes were then removed from the solution by filtration.

Anti-urate oxidase serum was then added to the solution (2 ul per 50 ul of translate) for 2 to 24 hours at 4°C. The IgG-peptide complexes were then removed with protein-A sepharose as above.

Immunoprecipitated peptides were prepared for SDS-polyacrylamide gel electrophoresis by resuspending the protein-A sepharose pellet in 20 ul of Laemmli sample buffer and heating at 100°C for 5 minutes. The peptides were then separated from the sepharose by filtration.

**Source of 20-Hydroxyecdysone**

20-hydroxyecdysone was obtained from two sources. Originally from Sigma, St. Louis, MO. and subsequently from Simes, s.p.a., Milano, Italy. Both 20-hydroxyecdysone

preparations were equally effective, but that obtained from Simes was significantly cheaper.

**Source and Preparation of Azaserine and DON**

Azaserine (carcinogenic) and 6-diazo-5-oxo-L-norleucine were both obtained from Calbiochem-Behring Corp, La Jolla, CA. 0.5M stock solutions in water were prepared and filtered through 0.22 micron filters. Unfiltered preparations had no inhibitory effects. Presumably, the compounds were contaminated by some organism which is capable of breaking down the molecules.

**Feeding Larvae on Defined Diets**

For the purposes of feeding larvae defined diets, 5 to 10 larvae were transferred from yeast food to 10-ml or 50-ml beakers containing cellulose powder (Whatman CF11) saturated with water or some defined nutrient solution. For 10-ml beakers, 160 mg of cellulose was saturated with 500-ul of solution. For 50-ml beakers, 650 mg of cellulose was saturated with 2-ml of solution.

To prevent evaporation and to prevent larvae from escaping, the tops of the beakers were sealed with parafilm and small pinholes were made for gas exchange.

**RNA Extraction from Malpighian Tubules**

Approximatly 500 Malpighian tubules were contained in 200-ul of Drosophila Ringer after isolation as described above. The frozen tubules in 200 ul of frozen Ringer were

transferred to a silanized 1.5 ml Eppendorf capsule. 25 ul of 10x extraction buffer (100 mM NaAc pH 5.1, 100 mM EDTA, 5% SDS) and 400-ul of phenol (80% phenol, 1% 8-hydroxyquinoline, 20% water) were added to the tubules. The mixture was incubated at 67°C for 20 minutes with vortexing at 2 to 5 minute intervals. This treatment dissolves the tubules. The mixture was then cooled on ice and 400-ul of chloroform was added. The mixture was vortexed and the aqueous and organic phases were separated by centrifugation for 1 minute in an Eppendorf centrifuge.

The aqueous phase was then transferred to a new silanized capsule and the organic layer was re-extracted with an additional 200-ul of 1x extaction buffer. The aqueous layers were then combined (400-ul) and 400-ul of phenol and 400-ul of chloroform were added sequentially with vortexing. The aqueous layer was again recovered after centrifugation and re-extracted in this manner about 5 times until the interphase between the organic and the aqueous layers was clear.

The RNA was then precipitated from the 400-ul of aqueous solution by the addition of 25 ul of 3M NaAc buffer (3M NaAc pH 6.0, 5 mM EDTA) and 1000-ul of ethanol at -80°C. The RNA was obtained by centrifugation in an Eppendorf centrifuge for 15 minutes at 4°C.

The RNA pellet was then washed twice with 500 ul of 3M NaAc buffer, redissolved in 400 ul of wash buffer (100 mM NaAc pH6.0, 5 mM EDTA) and precipitated at -80°C by the

addition of 1000 ul of ethanol.

PolyA$^+$ RNA was then obtained as follows: The RNA pellet was dissolved in 50-ul low salt buffer (10mM Tris-HCl pH 7.6, 1 mM EDTA, 0.1% SDS) and then 50 ul of 2x high salt buffer (low salt buffer made 0.8M NaCl) was added. The RNA was then loaded onto a micro oligo(-dT) cellulose column (100 to 200-ul bed volume). The eluate was collected, loaded onto the collumn once again, and the collumn was washed with 1.5-ml of 1x high salt buffer (low salt buffer made 0.4M NaCl).

PolyA$^+$ RNA was then eluted from the collumn in the first 400-ul of low salt buffer into a silanized Eppendorf capsule. 27 ul of 3M NaAc buffer and 1000 ul of ethanol were then added to the capsule and the RNA was precipitated at -80$^o$C.

It is important that all glassware and plasticware be silanized and RNAse free. All solutions were made with RNAse free distilled water treated with diethyl pyrocarbonate (50 ul per 1000 ml).

RNA was extracted from whole Drosophila larvae, pupae or adults in the same manner except that the initial extraction was made by combining 100mg of Drosophila, 200 ul of 1x extraction buffer and 400 ul of phenol in a Kontes 1.5-ml homogenizer. The homogenate was then transferred into a silanized Eppendorf capsule which was then incubated at 67$^o$C for 15 minutes with vortexing at 5 minute intervals. The rest of the extraction procedure was as described.

Large scale extractions were as described except that the volumes were increased as needed (eg. 100 grams of larvae would be combined in a homogenizer with 200 ml of 1x extraction buffer and 400 ml of phenol). Centrifugation for separation of phases was at 10,000 RPM at 4°C for 10 minutes in 50 ml polypropylene centrifuge tubes and RNA was pelleted after ethanol precipitation by centrifugation at 20,000 RPM at -20°C for 10 minutes.

**Northern Gel Analysis of RNA**

5-ug of ethanol precipitated PolyA$^+$ RNA was dissolved in 10-ul of a solution containing 50% deionized formamide, 2.2M formaldehyde and 1x gel buffer (20 mM Na-MOPS, 5 mM NaOAc, and 1 mM EDTA). The RNA was then denatured by heating at 65°C for 15 minutes. 1-ul of 10x loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol) was then added and the sample loaded on to a 1.2% agarose formaldehyde denaturing gel (1.2% agarose, 1x gel buffer, 2.2M formaldehyde).

After electrophoresis, the RNA was transferred from the gel to 0.45 micron nitrocellulose presoaked in 20x SSC by blotting with 20x SSC for 24 hours. The filter was then dried and baked in a vacuum oven at 80°C for 2 hours. After baking, the filter was washed with rubbing in 4x SEC (0.6M NaCl, 0.12M Tris-HCl pH 8.0, 0.008M EDTA).

The filter was prehybridized in 20-ml of buffer containing 8-ml 2.5x hybridization buffer (0.25M PIPES, 2.0M

NaCl, 0.25% Sarkosyl, 0.25% Ficoll, 0.25% PVP 40, 0.25% BSA-Pentex V), 10 ml formamide, and 2 ml of denatured salmon sperm DNA (10mg/ml). Before adding to the filter, the buffer was heated at 75°C for 20 minutes. The filter was prehybridized at 42°C for 4 hours.

For hybridization of the RNA with urate oxidase cDNA, the filter was incubated with 20 ml of a buffer containing 2 grams dextran sulfate (Pharmacia, MW 500,000), 8 ml 2.5x hybridization buffer, 10 ml formamide, 1 ml salmon sperm DNA (10mg/ml), and $10^7$ CPM (specific activity of 1.6 x $10^8$ CPM) of nick translated urate oxidase cDNA, pcDNAUO63. This buffer was first heated at 75°C for 20 minutes, cooled briefly and then added to the prehybridized filter. The filter was hybridized for 16 hours at 42°C.

After hybridization, the filter was washed twice in 2x SSC (1 liter contained 100-ml 20x SSC, 2.0 gm sarkosyl, 0.2 gm NaPPi) with rubbing at room temperature and then four times with 0.1x SSC (2 liters contained 10 ml 20x SSC, 1.0 gm sarkosyl, and 0.2 gm NaPPi) at 60°C. The filter was then dried and autoradiographed.

All solutions were RNAse free and the submarine gel aparatus was soaked in 0.5% SDS for one hour and then rinsed with RNAse-free water prior to electrophoresis.

**Alkaline Agarose Gel Electrophoresis**

The agarose was dissolved in 50mM NaCl and 1mM EDTA by boiling. The agarose was then cooled to 50°C and and the

gel cast. Once the gel had set, the gel was immersed in alkaline electrophoresis buffer (30 mM NaOH, 1 mM EDTA) and allowed to soak for 60 minutes so that the alkaline buffer soaks into the gel. The gel can not be cast in NaOH.

The DNA samples to be analyzed were then denatured by the addition of 1 volume of a 2x loading buffer (100mM NaOH, 2mM EDTA, 5% Ficoll, 0.05% bromocresol green) and loaded onto the gel. After electrophoresis, the gel was dried and autoradiographed.

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