

HORMONAL CONTROL OF PROTEIN GRANULE ACCUMULATION
IN FAT BODIES OF DROSOPHILA MELANOGASTER LARVAE

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DEDICATION

For Penny,

The birthday present she asked for.

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ABSTRACT

It has been previously shown that large granules of protein accumulate in the larval fat body of holometabolous insects shortly before pupation. In the butterfly Calpodex ethlius there is evidence that the protein in these granules is sequestered from the hemolymph and that their accumulation is controlled by the molting hormone, ecdysone. Evidence as to hormonal control of granule accumulation in Drosophila melanogaster is conflicting.

In this thesis it is shown that if ^3H -labelled proteins and ^{14}C -labelled amino acids are injected into D. melanogaster larvae at the time of granule formation, the proteins are incorporated into the granules but the free amino acids are not. The conclusion that all proteins in the granules are preformed hemolymph proteins is reinforced by the observation that injection of cycloheximide shortly before first appearance of the granules did not in any way interfere with their formation.

It is shown that injection of ecdysterone into early third instar larvae will induce precocious formation of granules. When fat bodies are incubated in vitro in media containing serum, addition of ecdysterone consistently causes granule formation. However, granules sometimes form in the absence of added hormone. Addition of 50 mg/ml or more protein consistently causes the formation of small granules, whether or not hormone is also present.

When fat bodies are incubated in media without serum, granules consistently appear in aerated media without addition of hormone. However, addition of hormone causes the formation of larger granules which appear earlier.

It is therefore concluded that in D. melanogaster ecdysone is sufficient but not necessary for granule formation.

Since the granules appear seventeen hours before pupariation, while ecdysone is secreted only four hours before pupariation, it is suggested that ecdysone is not involved in control of the natural appearance of the granules in D. melanogaster. The hypothesis is presented that the granules form spontaneously as soon as the juvenile hormone titer falls below a certain level.

In the course of this work it was incidentally noticed that injection of ecdysterone into early third instar larvae caused a syndrome which resembled a partial and abortive prepupal molt. No puparium was formed and the larvae died within a few days.

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MOLECULAR MECHANISMS OF STERIOD HORMONE ACTION

A Review and Speculation

Over the past decade, one of the most lively topics in the field of molecular biology has been the study of the mechanisms by which the concentrations of certain inducible enzymes are regulated in bacteria. It has been fascinating to watch as the brilliant early model of Jacob and Monod (1) was step by step confirmed, with only minor additions and modifications. The final, clinching proof was the recent isolation (2) and purification (3) of the repressor for the lac operon and the demonstration that it is active in vitro (4).

It is important, however, to temper our enthusiasm with caution by noting that the model does not explain the phenomenon of catabolite repression--the observation that addition of glucose to the culture medium transiently blocks synthesis of certain inducible enzymes, then permits synthesis only at a lower rate than is otherwise observed. This repression is relieved by addition of cyclic adenosine-3', 5'-monophosphate (cAMP) (5). Since the phenomenon is observed even in repressorless and operator constitutive strains (6), it is obviously outside the scope of the Jacob and Monod model. Rather, in some as yet unexplained manner it is involved with the more recently discovered promoter region, since strains in which the promoter has been deleted do not show catabolite repression (7, 8) while a point mutant in the promoter region is known in which exceptionally high concentrations of cAMP are required to relieve the repression (8).

Even in bacteria, it seems, there may be more than one way to regulate enzyme concentrations.

Nevertheless, the significant success of the Jacob and Monod model has naturally encouraged a search for analogous mechanisms in higher (eukaryotic) organisms. However, most work in bacteria has utilized as inducers nutrients which are present in the organism's natural environment in widely varying amounts, resulting in a varying need for the enzymes that metabolize or produce them. Since the "external environment" for the cells of a multicellular higher organism is the body fluids--of essentially unvarying composition--it is not surprising that this sort of substrate induction has been rarely found in these systems.

It is obvious, of course, that higher organisms have mechanisms for altering the metabolic activities of their cells. The most spectacular example of this is tissue differentiation, in which a single fertilized egg produces tissues with metabolic activities as different as those of brain, liver, and skin (or root, leaf, and meristem). But the changes occurring in differentiation are complex, and the evidence of experimental embryology suggests that the control mechanisms may be almost equally so.

Much attention has therefore been focused on action of hormones. These cause rather specific and often reversible changes in the metabolism of their target organs. It is known in many cases that increases in the concentration of definite enzymes are produced, and that these increases are dependent on RNA and protein synthesis. They are therefore analogous to enzyme induction in bacteria. The problem gains added interest from the association of hormones with certain cases of developmental tissue differentiation, as the thyroxine-

induced metamorphosis of the tadpole (9) and the complex interaction of insulin, cortisone, and prolactin in the maturation of mammary glands (10).

The greatest progress in elucidating the mechanisms of action of hormones has so far occurred with hormones of the polypeptide and catecholamine classes. Ironically, this progress has made these hormones less attractive to those seeking to understand intracellular regulatory mechanisms, since it appears that their primary action is exclusively at the cell membrane (11). The majority of the effects of these hormones are apparently due to changes in the intracellular concentration of cAMP resulting from the direct effect of the hormones on membrane-bound adenylyl cyclase. Some hormone effects are the result of cAMP interacting directly with enzymes, for example liver glycogen phosphorylase kinase kinase (12), but other effects--also due to cAMP--are true enzyme inductions requiring RNA and protein synthesis (13). However, very little work has yet been reported on the mechanism by which cAMP produces these inductions.

Many workers have therefore focused their attention on the steroid hormones. While there have been several reports that cAMP may be involved in the actions of these hormones (14), the evidence is quite strong that at least some effects are not mediated in this way. Most strikingly, cortisol induces tyrosine aminotransferase in hepatoma cells in which both adenylyl cyclase and cAMP are undetectable (15).

There has naturally been no lack of work on any hormone-target organ system, and indeed the greatest progress in isolating

steroid-binding proteins has come with the estradiol "receptors" of the uterus (whose physiological significance, however, remains to be demonstrated). However, most theorists have tended to concentrate on one or both of two systems: the induction of gluconeogenic enzymes-- especially tyrosine aminotransferase--in mammalian (rat) liver by glucocorticoids, and effects of the insect molting hormone (ecdysone), with particular emphasis on changes in the puffing patterns of the polytene chromosomes of dipteran salivary glands. While the types of experiments which can be done in these two systems are to some extent complementary, each contains ambiguities which make it difficult to obtain unequivocal support for any particular theory.

There are three principal theories which have been elaborated in sufficient detail to qualify as satisfying and testable hypotheses. They differ essentially in the site of action which they assume for the hormone. However, as will be noted, the primary effect with which they are concerned is different in each case, making them neither strictly comparable nor in the fullest sense mutually exclusive. They are primarily associated with the names of Peter Karlson, Heinrich Kroeger, and Gordon Tomkins.

Karlson: Direct Action at the Gene

This theory (16) is a relatively straightforward application to animal cells of the principles developed by Jacob and Monod for bacterial cells. The hormone penetrates to the nucleus where it interacts with a specific chromosome-bound repressor, relieving the inhibition of RNA synthesis which the repressor normally produces. (Positive

interaction with a specific promoter is not explicitly considered.) The "turning on" of mRNA synthesis at these loci is assumed to produce the 50-60% increase in overall RNA synthesis usually observed after hormone administration (17, 18), and the resulting rapid increase in specific mRNAs leads to a rapid increase in the concentrations of the proteins for which they code.

There are several critical features of this model which in principle are experimentally testable. First, since the increase in RNA synthesis is a direct effect of the hormone, it should occur within a very short time after hormone administration. (A variant of the model would propose that the hormone directly activates only a small number of genes, whose products then activate a much larger number. The principal rise in RNA synthesis might thus be somewhat delayed, but should still precede the inductions for which the hormone is responsible. Such sequential activation has been demonstrated for the action of ecdysone on Chironomus salivary glands (19).)

Furthermore, if this were the only mechanism of action of the hormone--as is sometimes implied--there should be no effect of the hormone prior to an increase in RNA synthesis. This is definitely not true for the effects of estrogen on the uterus. In less than one minute the hormone increases the concentration of cAMP (again!) and causes the release of endogenous histamine and dilation of the blood vessels of the organ (20); the dilation of the blood vessels and accompanying uptake of water by uterine cells are probably due to the released histamine. This increased uptake of water is exactly paralleled by an increased uptake of RNA precursors, which in turn accounts for

essentially all of the apparent increase in RNA synthesis observed in the first six hours after hormone administration (21). There is a true increase in RNA synthesis at later times. It should be noted, however, that the increased uptake of water can be prevented by puromycin (22) and partially inhibited by Actinomycin D (23).

While this example shows clearly that steroid hormones exert effects in ways other than that considered in the Karlson model, the prompt rise in RNA synthesis in liver in response to cortisol (17) and the quick puffing in a few loci in Chironomus salivary glands (24) are compatible with applicability of the model in other cases.

The second prediction of the Karlson model is that hormone should increase RNA synthesis in isolated nuclei or even isolated chromatin. This latter prediction seems not to have been confirmed; there has been no reported case in which the template activity of isolated chromatin has been increased solely by the addition of hormone in vitro (but see the discussion below of the Matthyse and Phillips experiment). However, there have been several reports of increased RNA synthesis when hormone was added in vitro to isolated nuclei. According to Dukes and Sekeris (25), cortisol caused a 70% increase in RNA synthesis by isolated rat liver nuclei. This is accompanied by an increase in the activity of "aggregate RNA polymerase" (26) and of the template activity of the chromatin isolated from these nuclei (27).

These results have been confirmed by Ohtsuka and Koide (28). However, Drews and Bondy (29) point out that the hormone concentrations used (25×10^{-6} M) are some three orders of magnitude larger than physiological, and report that they find no stimulation with 10^{-8} M cortisol; they further point out that there is extensive RNA degradation in isolated nuclei, which they feel casts doubt on the significance of the

results of Dukes and Sekeris. MacGregor and Mahler (30), using nuclei prepared in such a way as to minimize RNase activity, also find no stimulation of RNA synthesis by hormone added in vitro.

Before passing from this point, it seems appropriate to consider in some detail the significance of the increase in RNA synthesis seen after hormone administration, either in vitro or in vivo. The contention of the Karlson group is that this represents the synthesis of certain (perhaps many) hormone-specific mRNAs. They have sought to buttress this opinion by showing that the increase is greatest for the rapidly labeled, "DNA-like" RNA extracted by phenol at 60° but not at 50° (31) and that nuclear RNA shows greater stimulatory activity in an in vitro protein synthesis system after hormone administration (32). However, since ribosomal precursor RNA stimulates protein synthesis in vitro, and since it is known that over 50% of the rapidly labeled nuclear RNA is degraded without ever leaving the nucleus (in fact, Britten and Davidson (33) base their theory of gene regulation on this observation), these points are insufficient to prove the contention.

This contention would draw support from the demonstration that production of mRNA for an inducible enzyme is increased following hormone administration. Sekeris and Lang (34) have sought to do this using RNA extracted from epidermal cell nuclei of 7-day or 8-day old Calliphora larvae. Since ecdysone is secreted during the 8th day of larval life in this species, they refer to the 8-day cells as induced and the 7-day cells as uninduced. They report that the RNA from induced, but not uninduced, cells will cause formation of the inducible enzyme dopa decarboxylase in an in vitro protein synthesis system. The

enzyme was detected by the conversion of dopa to dopamine, identified chromatographically.

We now realize, however, that initiation is not normal in in vitro protein synthesis systems from higher organisms, and that therefore production of functional protein is not to be expected. The fact that no metabolite of dopa other than dopamine was found must also be considered surprising. It is therefore necessary to treat these results with great reservation.

The third prediction of the Karlson model is that chromatin-bound repressors will be found which interact with hormone. The most significant results along these lines have come with the "estrogen receptors" of the uterus. These proteins exist in the cytoplasm as molecules with a sedimentation coefficient of 9s. On addition of radioactive estrogen, the hormone first binds to these cytoplasmic receptors, then disappears from the cytoplasm and is found bound to the chromatin. By use of 0.3 M KCl the hormone can be extracted from the chromatin as a complex with a 5s protein (35); it was later found that 0.3 M KCl converts the 9s protein to a 5s protein (36). However, addition of hormone directly to isolated nuclei does not result in binding of hormone to chromatin.

While these proteins have many of the properties to be expected of estrogen receptors, it has not yet been conclusively demonstrated that they actually play any role in the mediation of hormone action. In another case the evidence is much clearer. Mathysse and Phillips (37) have found that the template activity of chromatin from tobacco callus can be increased by the addition of auxin plus a protein from the

nucleus of tobacco or other plants. This protein is found in the nuclei only if they were isolated in the presence of auxin. Neither the auxin nor the protein alone had any effect.

Auxin is not, of course, a steroid, though they are similar to the extent of not containing nitrogen. Nevertheless, demonstration that one hormone is capable (under proper circumstances) of acting directly on chromatin makes it more believable that others do also.

Kroeger: Action at the Membrane

This theory was developed primarily to explain the effects of juvenile hormone and ecdysone on the puffing patterns of the salivary gland chromosomes of dipteran larvae. It seems to have gone through several variations, but all emphasize hormone effects on ion pumps in the membrane.

In the original form of this theory the factor controlling the activation of different groups of genes was thought to be the intracellular Na^+/K^+ ratio. Juvenile hormone was presumed to increase this ratio and ecdysone to decrease it. This idea was based on experiments in which salivary glands of Chironomus thummi were explanted into saline solutions of varied Na^+ and K^+ content (38). Kroeger felt that the puffing pattern which he observed in the high- Na^+ medium was identical to the pattern observed on wounding an intact animal; he interprets this as an exaggeration of the normal "juvenile" pattern. The pattern in the high- K^+ medium was asserted to be identical to that normally produced by ecdysone. Unfortunately, these experiments were fatally flawed, since he replaced NaCl with KCl on a weight-for-

weight basis, rather than a mole-for-mole basis. He was therefore changing not only the ionic composition but also the ionic strength of the medium.

Clever was therefore able to argue that the puffing changes were due solely to the changes in the ionic strength and that they were therefore non-specific. He never found any difference in the puffing patterns of sister salivary glands of Ch. tentans incubated in equimolar NaCl or KCl solutions, though definite changes in puffing pattern occurred as the ionic strength was increased (39). He also found characteristic puffing patterns in various other media, but none resembled that due to ecdysone.

These experiments were countered by those of Lezzi (40) who was able to follow changes in the puffing pattern of isolated nuclei from the salivary glands of both Ch. thummi and Ch. tentans. He found that a specific puff--previously shown to be induced by ecdysone--appeared when the nuclei were incubated in 0.14 M KCl but not when they were incubated in 0.14 M NaCl. These same effects were observed with whole salivary glands of Ch. thummi but not of Ch. tentans. He concluded that glands of this latter species were impermeable to the ions, thus explaining Clever's negative results. These experiments were further extended by the observation that one of the four Balbiani rings (giant puffs) of Ch. thummi salivary gland chromosomes was specifically activated when the nuclei were incubated in high-Mg⁺⁺ medium (41).

Lezzi's interpretation of Kroeger's theory therefore seems to be that specific groups of genes are activated by specific ions (or possibly

by other substances) whose entry into the cell has been facilitated by the hormone. While he never draws the analogy, he appears to view K^+ and Mg^{++} as functioning as "second messengers" in a manner similar to that of cAMP.

Kroeger has presented one additional, and very striking, piece of evidence for his view that the primary site of hormone action is at the membrane. He measured the membrane potential of explanted Ch. thummi salivary glands (42), finding that this potential was higher (more negative) in the later developmental stages. By the use of reference solutions with and without potassium he was able to show that an increase in intracellular K^+ accounted for part, but not all, of the observed difference. He was also able to show that the degree of development of the "wounding pattern" following explantation was correlated with the degree to which the potential fell during this period.

But most convincingly, he was able to show that addition of ecdysone to the medium caused a sharp rise in potential. Furthermore, a particular ecdysone-sensitive puff appeared in 13 out of 14 glands in which the potential reached -35 mV, but in 0 out of 4 glands in which it did not. An increase in potential was measurable within 1 minute of the addition of hormone.

These results are complemented by those of Baumann (43) on Galleria salivary glands. He found that juvenile hormone caused a pronounced decrease in the membrane potential. However, since he found that this was preceded by a decrease in membrane resistance, he concluded that the latter was the primary effect, and that the potential decrease was secondary. This is furthermore compatible with the results

of Ito and Loewenstein (44) who injected ecdysone into Ch. thummi and measured the membrane potential in vitro 1 to 5 hours later. They found the hormone had no effect on the potential under these circumstances (so there was no long-term effect on the ionic composition of the gland) but that there was an increase in the electrical resistance of the nuclear membrane.

There has been one serious attempt by others to test the effects of ions on gene activation. Congote et al. (45) examined the effects of ecdysone, juvenile hormone, 50 mM NaCl, and 50 mM KCl on RNA synthesis in isolated nuclei from the fat bodies of Calliphora erythrocephala. The synthetic ability of different preparations of nuclei was unfortunately extremely variable, so that it was necessary to use statistical analysis to determine the significance of the various results. It is clear, however, that ecdysone and NaCl increase the rate of RNA synthesis, and probable that juvenile hormone does (borderline statistical significance). There was no significant effect due to KCl. However, the nuclei were isolated in medium containing 25 mM KCl. It is conceivable that a potential KCl effect has occurred irreversibly at the time, so that KCl in the synthesis medium could have no further effect. For example, if KCl dissociates repressors from certain specific loci on the chromosomes, then the presence of KCl in the isolation medium will cause their permanent loss.

The interactions between the different hormones and ions in this experiment were interesting. The stimulations due to ecdysone and NaCl were essentially additive, but there was no stimulation at all when both ecdysone and juvenile hormone were present simultaneously.

The stimulation by juvenile hormone was also abolished by KCl, which had no effect on the stimulation due to ecdysone. In the presence of both juvenile hormone and NaCl, the rate of synthesis was quite similar to that seen with either alone; the authors prefer to interpret this as indicating that NaCl has abolished the stimulatory effect of juvenile hormone.

As the authors point out, these results imply that there are two mechanisms by which RNA synthesis can be increased, one directly sensitive to the hormones and the other sodium dependent. As mentioned above, they may have overlooked a potential potassium dependent mechanism. They consider that the effects of juvenile hormone are abolished both specifically by ecdysone and non-specifically by high ionic strength (both NaCl and KCl).

Given a few ad hoc, but not unreasonable, assumptions it seems quite possible to reconcile the effects of the ions in these experiments with Kroeger's theory, either in its original form or as modified by Lezzi. An idea of this type is developed more fully on pages 21-24.

Tomkins: A Posttranscriptional Effect

Tomkins' approach to this question has differed from that of Karlson and Kroeger, in that the latter two authors have started from explicit theories of hormone action, for which they have then sought experimental confirmation. Tomkins, on the other hand, started with an experimental analysis of a particular system. In the past few years, however, his interpretation of his results has become sufficiently broad and detailed to qualify as a theory of steroid action--one which

he believes to be of general applicability.

The biological material with which Tomkins has worked has been HTC cells, the name which he has given to a minimal deviation hepatoma adapted for growth in tissue culture. Glucocorticoids induce increased levels of tyrosine aminotransferase (TAT) in these cells (46), although tryptophan oxygenase which is also induced in normal liver is not induced in this system. There is also no increase in overall protein (46) or RNA (47) synthesis, two of the most striking effects in the normal response and the basis for most of Karlson's work. It should also be noted that the induced TAT levels in these cells are lower than the basal levels in normal liver (48).

Tomkins' theory stems basically from one simple, paradoxical observation: while addition of Actinomycin D (Act) to the medium prior to addition of hormone prevents induction, as expected, addition of inhibitor to cells in which, following hormone administration, the enzyme concentration has already reached its maximum level leads to a still further increase in enzyme activity, a superinduction (49). Even the addition of Act to cells never exposed to hormone leads to a small increase in TAT levels (50).

Reel and Kenney (51), having found that Act inhibited degradation of TAT, proposed that this could account for the superinduction phenomenon. However, it has since been found that Act prevents only the enhanced degradation consequent on a step-down to a medium less rich in nutrients (52), and superinduction is not dependent on a nutritional step-down. Accordingly, Tomkins was led to suggest an alternative hypothesis--that translation of the mRNA for TAT is normally

prevented by a protein of very short half-life, synthesized on a mRNA of very short half-life. When Act is added, the repressor will disappear faster than the TAT messenger, leading to increased synthesis of TAT. Furthermore, he proposed that the hormone might act primarily as an antagonist of this repressor.

Very interesting observations on synchronized HTC cells, summarized in Figure 1, have led to further extension and refinement of the model. Briefly, during the last 10 hours of G1 and all of S phase, the cells behave "normally": addition of hormone induces TAT synthesis which declines when hormone is removed (53); both effects are prevented by Act. During the remainder of the cell cycle, however, the situation is quite different: addition of hormone to non-induced cells does not increase the level of TAT, while removal of hormone from induced cells does not cause the TAT level to fall (54). (Tomkins refers to this latter phenomenon as "constitutive synthesis". As this usage of the term differs significantly from the established usage in bacterial genetics it could well confuse the casual reader.) Act has no effect during this non-inducible period.

Tomkins and his coworkers have therefore presented the following detailed model of cortisol induction of TAT (55): the system consists of two genes, the structural gene for TAT and the gene coding for the posttranscriptional repressor. Both genes are transcribed continuously during the inducible period and are completely repressed during the non-inducible period--this repression is not affected by hormone. The repressor prevents the translation of the TAT messenger, and the only effect of the hormone is to antagonize this action. If the

Hour		1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24																							
Phase of cell cycle		G2			M		G1												S						
Uninduced Cells	Effect of hormone	None					Induces TAT																		
	Effect of Act	None					Prevents induction Causes small increase in TAT levels																		
Induced Cells	Effect of removing hormone	None					TAT declines																		
	Effect of Act	None					Superinduces Prevents decline following hormone removal																		
	TAT messenger stability	Stable					Half-life 3 hours																		

Figure 1. Summary of some results obtained by Tomkins using synchronized HTC cells.

repressor prevents translation of the TAT messenger it is degraded with a half-life of three hours; otherwise this messenger is completely stable while, as indicated, both the repressor and its mRNA are extremely labile under all conditions.

Tomkins was at first uncertain whether the repressor directly prevented translation of the TAT messenger, or whether it rather degraded it or prevented its transport to the cytoplasm. The evidence in favor of the first possibility is that Act can still produce superinduced TAT levels even if it is added following removal of hormone, while the TAT level is declining (55). This demonstrates that even when the amount of active TAT messenger is falling there is a pool of repressed but potentially active messenger.

Since this conclusion requires that the repressor be located in the cytoplasm, it makes it somewhat unlikely that the hormone interacts directly with the repressor as all the specific corticosteroid binding sites so far reported are in the nucleus (56). Tomkins mentions evidence that there are also specific binding sites in the cytoplasm, but this evidence is not yet published.

Tomkins believes that cortisol has no effect on the transcription of TAT messenger. It is true that he has been unable to find evidence for such an effect in HTC cells; nevertheless, it remains possible that more sensitive methods would reveal such evidence. Even if the assertion is true for HTC cells it would appear extremely rash to extend it to normal liver, as Tomkins apparently does. HTC cells have obviously lost many of the capacities normally present in liver. One of these is the capacity to respond to cortisol by an overall increase in RNA and

protein synthesis. It would not be unreasonable to think that these cells might also have lost the capacity to increase selectively the synthesis of specific RNAs. The lower levels of TAT in HTC cells certainly suggests that the mechanism for synthesis of the enzyme is in some way abnormal.

Some Concluding Remarks and a Point of View

In attempting an overall assessment of the various theories, it would be well to start by pointing out that a modification of Karlson's theory appears necessary. There has been no evidence whatever to support his original assumption that the hormone interacts simply and directly with a chromatin-bound repressor, as bacterial inducers do. Rather, the evidence--especially of the Matthyse and Phillips experiment and of the estrogen receptors--is that the hormone interacts with a soluble protein which mediates its action on nuclear RNA synthesis. The evidence is not incompatible with the assumption that this hormone-protein complex acts by neutralizing repressors, but I prefer the alternative assumption that it acts either as a specific promoter for the transcription of certain genes or as a non-specific enhancer of RNA synthesis (or both). The fact that Matthyse and Phillips were able to obtain their results at saturating concentration of E. coli RNA polymerase could be adduced as an argument against this possibility, but I do not feel that we understand the factors governing the interaction of this enzyme with the chromatin of higher organisms sufficiently well for the argument to be conclusive.

Given this modification, the principal difficulty for the Karlson model remains the relationship between observed increases in RNA

and protein synthesis and the induction of specific enzymes. It is simply incredible that synthesis of the dozen or so (at most) enzymes known to be induced in any given case could require an increase of 50-70% in the RNA synthesis of the whole cell. Most of this, and of the corresponding increase in protein synthesis, simply must represent a general speeding up of cellular metabolism. Therefore, while it is not improbable that there are also specific increases in transcription of certain genes, we must conclude that no convincing evidence to support this assertion has yet been presented.

Turning now to Kroeger's theory, it is clear that in its original form it is untenable. If ecdysone decreases the Na^+/K^+ ratio and juvenile hormone increases it, then the two hormones should negate each other when they are present simultaneously. In fact, of course, it is precisely the presence of both hormones which leads to a larval molt.

Lezzi's alternative version of the theory, where ions function as "second messengers", is more acceptable, but the evidence in its favor is far from conclusive. His experiments, to be sure, clearly show that high concentrations of K^+ or Mg^{++} can activate specific loci. This specificity is not as surprising as it might at first seem, since a number of enzymes are known to be activated specifically either by Na^+ or else by K^+ (or NH_4^+) ions (57). However, the physiological significance of Lezzi's observations is not clear. While his solutions were at physiological ionic strength, K^+ (or Mg^{++}) was the only cation present, a situation which is never even approximately true in vivo. Furthermore, discounting Kroeger's original experiments, positive results are reported for only one locus, and negative (control) results for

extremely few. It is therefore difficult to feel really confident that K^+ does indeed mimic the effect of ecdysone on the puffing pattern.

It is, however, quite important to note that Kroeger's experiments on membrane potential prove conclusively that something happens at the membrane, and that this is too fast to be secondary to something at the nuclear or cytoplasmic level.

Tomkins' work is experimentally the most clear cut of the three. There seems no alternative to the conclusion that there exists a cytoplasmic repressor of translation--the ability to "rescue" repressed but undegraded messenger by the addition of Act during the period when enzyme concentrations are falling is particularly convincing.

But Tomkins' theory is weakest precisely where the others are strongest. They emphasize immediate actions of the hormones, but are short on concrete evidence as to how this affects the machinery for synthesis of a particular enzyme. Tomkins has good evidence as to how the synthetic machinery is affected, but is hazy as to how the hormone interacts with it. In fact, he has never presented the slightest evidence that the repressor he is studying plays any role at all in the induction of TAT by cortisol.

So who's right? In some sense they all must be. It is absolutely certain that ecdysone has some effect at the membrane, and the evidence that specific ions activate RNA synthesis, while slightly shaky, is by no means negligible. It is clear that auxin--in conjunction with a supernatant protein--can increase the template activity of chromatin, and there is strong evidence that steroids do too. And it is virtually certain that there is a cytoplasmic inhibitor of the translation of TAT

messenger.

So we are led to the conclusion that the same hormone acts in a number of ways. This may at first seem surprising, but it is not. In fact, we should have been surprised if it were not so.

Let us consider carefully--instead of carefully ignoring--the way in which ecdysone and juvenile hormone interact during the life cycle of the insect. When both are present, the animal molts into another larval instar, but when ecdysone is present while juvenile hormone is absent the animal forms a puparium, then undergoes prepupal and pupal molts. The response to ecdysone must therefore differ depending on the concentration of juvenile hormone.

In this light let us consider Figure 2, a hypothetical model based largely on the experiments of Kroeger and of Congote et al. with some speculative but common-sense additions. It should be noted that this is a functional, rather than a mechanistic model. For the sake of mechanistic concreteness we may imagine that the various inducers "activate" a gene by allosterically modifying an ineffective promoter protein into an effective one, and that other effectors "block this activation" either by preventing combination of the inducer and the promoter or by rendering this combination ineffective in causing the requisite allosteric transition.

Figure 2 is in several other respects obviously oversimplified. In the first place, only gene activation is considered; the possibility that certain genes may be repressed by hormones is ignored, as are translation-level mechanisms and enzyme activation. In the second place, only three groups of genes are considered: those involved in

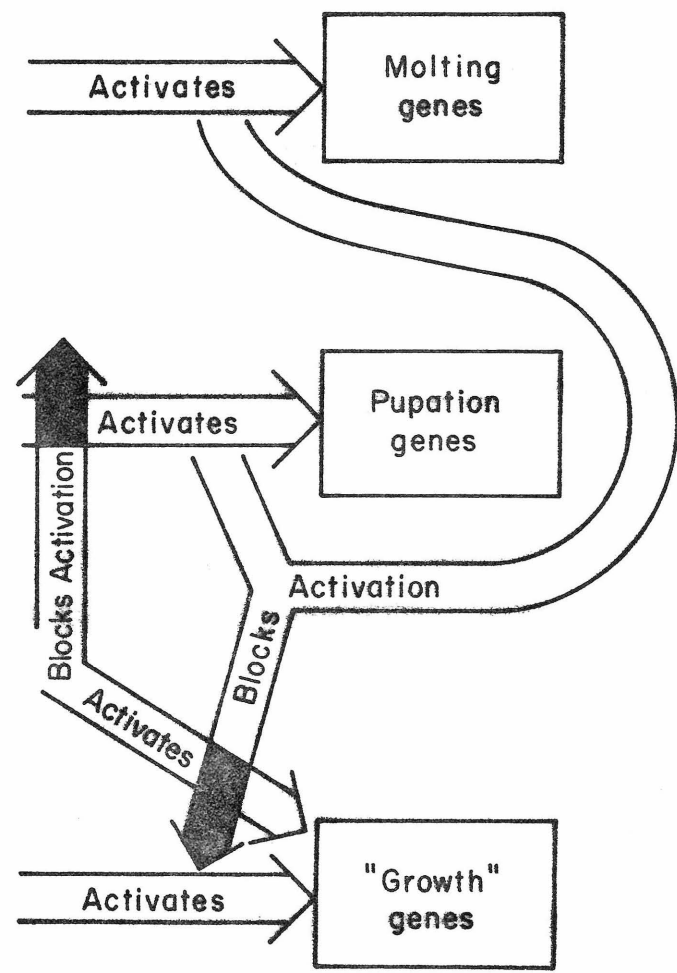
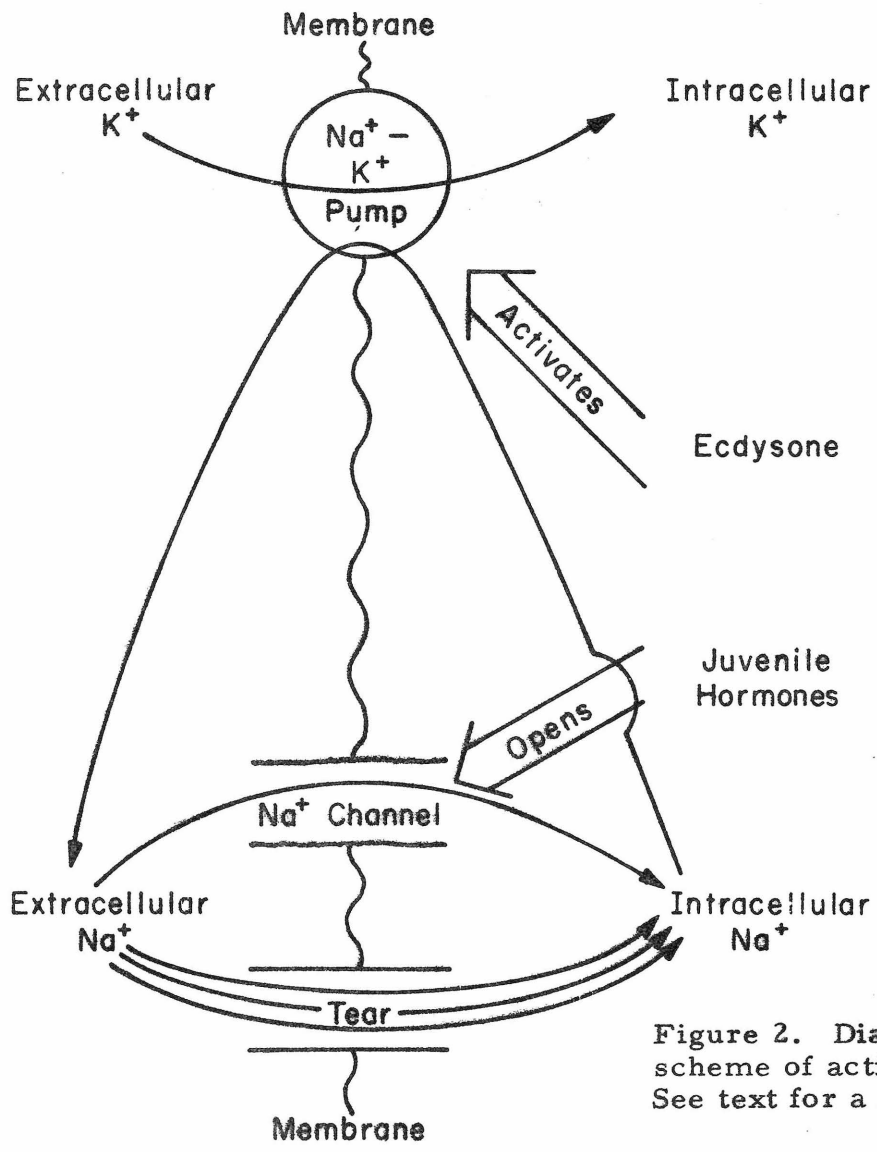


Figure 2. Diagrammatic outline of a hypothetical scheme of action for ecdysone and juvenile hormone. See text for a fuller explanation.

molting, those involved in pupariation, and "growth" genes. These latter are involved in the processes of rapid cell growth taking place during the 1st and 2nd and early 3rd larval instars, and are involved to an even greater extent in the recovery processes following wounding.

The involvement of Na^+ in the control of these "growth" genes can be easily rationalized on the basis of this dual requirement for their products. During the period of normal growth they are activated directly by JH and are also activated by Na^+ ions to which JH has rendered the cell membrane increasingly permeable. When the animal is faced with the metabolic demands of molting it becomes desirable to divert resources from growth processes. This is accomplished, inasmuch as ecdysone interferes with the direct activation of the growth genes by JH while the intracellular Na^+ concentration is lowered by the increased activity of the $\text{Na}^+ - \text{K}^+$ pump. The increased K^+ concentration also prevents direct activation of these genes by JH; this can be viewed as a double-safety mechanism.

On the other hand, wounding constitutes a high-priority demand which must take precedence over all others, without regard to position in the molting cycle. In fact, any tear in the cell membrane will flood the cell with Na^+ to an extent greatly exceeding the capacity of the $\text{Na}^+ - \text{K}^+$ pump to remove it. This will result in turning on the "growth" genes in a manner which cannot be blocked by ecdysone.

The above analysis actually does not reveal any reason why it should be necessary for JH to directly activate the "growth" genes, although this is required by the data of Congote et al. This may again be a double-safety mechanism, or it could possibly indicate the existence

of situations in which JH would not lead to increased intracellular Na^+ concentrations.

This illustrates the control of one set of genes by two different effectors. Conversely, ecdysone controls two different sets of genes. It activates the pupation genes directly while, by activating the $\text{Na}^+ - \text{K}^+$ pump, it raises the intracellular K^+ concentration and thus indirectly activates the molting genes. This creates a situation in which JH can block the effect of ecdysone on the pupation genes without interfering with its effect on the molting genes.

Yet another application of this principal can be appreciated by a consideration of the effect of several different hormones on the same enzyme. I will take tyrosine aminotransferase as my example. This enzyme is known to be induced by both cortisol and glucagon; I would also suggest that tyrosine must play a permissive role in these inductions, though there is no experimental evidence of this. Nevertheless, tyrosine is a semi-essential amino acid, and if it is in short supply then induction of TAT--diverting tyrosine into the gluconeogenic pathway--would not be advantageous.

My model proposes the existence of a specific promoter protein for the synthesis of the TAT messenger. It is assumed that synthesis of this promoter requires an adequate supply of tyrosine--it is probably relatively rich in tyrosine. In addition its synthesis may require at several points a rare tyrosine-tRNA whose corresponding synthetase has an unusually high K_m for the amino acid. This promoter is assumed to act ordinarily with rather low efficiency; the effect of cortisol is to convert it to a high-efficiency state, thereby increasing the rate of

synthesis of the TAT messenger.

This hypothesis is in accord both with the observation that protein synthesis is not necessary for cortisol to cause an increase in TAT messenger (58)--so that the hormone does not simply increase the synthesis of promoter--and with the observation that accumulation of TAT messenger can be detected within 30 minutes, while increased TAT synthesis is not detectable for two hours (59). The intervening hour and a half are presumably devoted to intranuclear processing of the newly synthesized mRNA.

At this point I would like to add a detail to the model which is required neither by experimental evidence nor by any compelling functional argument, but which I find intellectually satisfying. I propose that both TAT and its translation repressor are synthesized on a single polycistronic mRNA. Ribosomes are assumed to add to this messenger only at the TAT end, so that this provides a feedback loop whereby the repressor regulates its own synthesis in a manner automatically coupled to synthesis of TAT.

It should be noted that the Act data still require that the portion of the messenger coding for the repressor be much less stable than that coding for TAT. While there is no obvious mechanism for accomplishing this, it is no more mysterious than the many other examples of differential stabilities of mRNAs. Putting the two messages in one continuous strand makes it neither easier nor harder to explain.

This proposal implies that cortisol increases the amounts of both TAT messenger and repressor, but the larger amount of messenger operating at the same moderate efficiency will obviously produce

more TAT. However, it is clear that complete removal of repressor, as by Act will have a much larger effect in the induced state. This seems to be the case.

It has been known for several years that glucagon also induces TAT (60). This is presumably mediated by cAMP, which has also been shown to induce the enzyme (61). There has been a recent report that in Rueber hepatoma cells in culture, glucagon causes an "immediate" increase in TAT levels which is insensitive to Actinomycin D and is synergistic with cortisol induction (62). All this is exactly what would be expected if cAMP antagonized the effect of the translational repressor which Tomkins has been studying; I propose this as the mechanism by which glucagon induces TAT. Since HTC cells lack cAMP this mechanism naturally could not be physiologically triggered in them.

In summary then, we must recognize that cells of higher organisms are exposed to a very complex and constantly changing hormonal and nutritional environment. The appropriateness of the induction of any given enzyme is not governed solely by the presence or absence of any one hormone, but by the interrelationships of several factors. It would seem reasonable that these relationships can be most satisfactorily taken into account if synthesis of the enzyme is controlled at several different levels.

It is conversely equally true that the various enzymes controlled by a given hormone must respond differently to the other variables in the milieu. We should therefore clearly expect that the hormone will use different mechanisms and different secondary messengers in controlling them.

The "one hormone--one mechanism of action" hypothesis must therefore be decisively rejected. Any one hormone must act on any given target organ, and perhaps even on a single genetic locus, in a number of different ways.

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THE FAT BODY AND ITS GRANULES: BACKGROUND

Naming this insect organ the "fat body" is in some ways misleading, even though it does store large quantities of fat. It has been more aptly compared to the vertebrate liver. Like the liver, it is the animal's principal storage depot for glycogen and it synthesizes or breaks down these glycogen reserves as needed to maintain the optimum level of blood sugar--trehalose in the case of insects. It is also probably involved in the homeostatic regulation of other metabolites. It is even, like the liver, involved in the metabolic inactivation of steroid hormones (1). For a general review of metabolic pathways in the fat body see reference (2).

It has been recognized since the turn of the century that the larval fat body of some insects contains granules of protein (3). At least since the work of Bishop (4) on Apis mellifera in 1922 (several earlier references by other authors were not available to me) it has been clear that these granules usually appear shortly before pupation. There appears to have been no experimental work designed to elucidate the function of the granules, but it is usual and natural to assume that they are reserves of protein for use during metamorphosis. As the larval fat body of holometabolous insects breaks down and autolyzes shortly after pupation (the adult fat body arises from imaginal disc cells) it seems reasonable to assume the granules would be available for this purpose. In at least one instance it has been reported that granules are absent from the fat body of a hemimetabolous insect (5), where on this assumption they would not be needed. They seem to be widespread, perhaps universal, among holometabolous orders.

In 1963, in the course of a light and electron microscopic investigation of the developmental cytology of the fat body, von Gaudecker (6) demonstrated that Drosophila melanogaster is no exception to this rule. The granules she observed were up to 6μ across and represented approximately 25% of the cell volume when maximally developed. She first observed small, mitochondrion-sized granules, which she interpreted as precursors of the larger granules, at 20 hours after the second larval molt (28 hours before puparium formation). The granules increased in size and number up to 45 hours after the molt. She occasionally found both residues of mitochondria and stacks of rough endoplasmic reticulum within the granules, suggesting that they had formed by the enclosure of areas of cytoplasm within bounding membranes. She interpreted the original mitochondrion-sized granules as being in fact altered mitochondria.

The granules were also noticed, apparently independently in the course of a similar investigation, by Butterworth et al. (7). Since the time at which the granules appeared suggested to them that they might be induced by ecdysone, they tested this possibility by implanting fat bodies from 2nd instar larvae into the abdomens of adult male hosts. The fat bodies survived quite well in this milieu, but granules--as hypothesized--did not form unless ring glands from late 3rd instar larvae were implanted as a source of ecdysone.

The simple picture was clouded, however, when Butterworth and Bodenstein (8) implanted the fat bodies into adult female hosts. Granules formed without any necessity of added ring glands! This was dependent on the presence of a functional ovary, as granules formed

neither in ovariectomized females nor in animals of the fes (female sterile of Bridges) genotype in which the ovary is non-functional. Implantation of wild-type ovaries along with the fat bodies into fes females permitted normal granule formation. However, when ovaries were implanted with the fat bodies in male hosts, granule formation was minimal at best; careful examination of the photographs has failed to convince me that the tiny specks seen bear any necessary relationship to true granules.

Since the corpus allatum (source of juvenile hormone) is hypertrophied in the fes mutant--though there is no agreement as to whether this indicates a high or low concentration of hormone (9, 10)--two to three corpus allatum-corpora cardiaca complexes from D. virilis were implanted into male hosts along with the fat bodies. In six out of seven cases granule formation was again minimal; in the seventh case it was reported as moderate. Butterworth and Bodenstein nevertheless concluded that both corpus allatum and ovary are active in inducing granules in male hosts.

These results can be illuminated by a comparison with the elegant work of Locke and Collins (11) on apparently similar granules in the butterfly Calpodex ethlius. They distinguish three different types of granules. At the beginning of the period of granule formation mitochondria are isolated by paired membranes to form bodies which fuse into large autophagic vacuoles. The mitochondria soon autolyze and the vacuoles disappear. At the end of the period of granule formation, endoplasmic reticulum is similarly isolated in autophagic vacuoles; autolysis of these is apparently incomplete.

These authors interpret their electron micrographs as indicating the following sequence of events in the formation of the true protein granules: protein is sequestered from the hemolymph in pinocytotic vesicles which then coalesce into multivesicular bodies. Prior to the time of granule formation these bodies are joined by microvesicles functionally analagous to lysosomes, resulting in digestion of the protein, but after initiation of granule formation this no longer occurs, so that continued fusion of pinocytotic vesicles results in large, permanent granules of protein.

This hypothesis implies that, contrary to the assumption of most previous authors, the protein in the granules is sequestered from the hemolymph rather than being synthesized in situ. This conclusion is supported by the following lines of evidence: (1) The rate of protein synthesis in the fat body is greatest 60 hours prior to the beginning of granule formation; by the time the granules are being made the rate of protein synthesis is less than half the maximum rate. (2) The concentration of protein in the hemolymph quadruples between the 66th and 156th hours of the last larval instar; between the 156th hour--when formation of granules begins--and pupation it drops to less than half its maximum value, as would be expected if it were being sequestered by the fat body. (3) When ^3H -tyrosine or ^3H -leucine is injected at the time of granule formation, radioautography reveals no significant concentration of grains over the granules relative to the rest of the fat body (12). (4) Most convincingly, when plant peroxidase is injected into the hemolymph it is incorporated into the granules in enzymatically active form.

The time at which granules first become visible in Calpodes is also the time at which the prothoracic gland is known to secrete ecdysone. Collins (13) therefore sought definite proof that ecdysone induces the granules in this species. Her primary technique for this purpose involved the use of larvae ligated either in the thoracic region or directly behind the head. In the former case the posterior portion of the animal is cut off from its normal source of ecdysone and does not pupate; in the latter case the ecdysone titer is normal but exposure to the brain hormone is minimal (some exposure is necessary to activate the prothoracic gland).

The results were clear. The posterior portion of the fat body of the animals ligated in the thoracic region did not form granules unless synthetic ecdysone was injected. The fat body of the animals ligated behind the head did form granules. Since the protein content of the hemolymph of these latter animals was half that of animals ligated at the thorax, these experiments also demonstrated that an elevated protein concentration was not necessary for granule formation. In Calpodes, ecdysone appears to be the only significant influence controlling granule formation.

This thesis will therefore be devoted primarily to the elucidation of two questions: are the proteins in the granules of Drosophila melanogaster, like those of Calpodes, primarily sequestered hemolymph proteins, and what is the role of ecdysone in the induction of the Drosophila granules?

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MATERIALS AND METHODS

Stocks and Culture Methods

The Oregon-R wild type stock maintained in this laboratory was used throughout these experiments. The larvae were cultured by the large scale method described by Mitchell and Mitchell (1).

Materials

4, 5-³H-L-leucine (58.3 C/mmole) and uniformly labeled ¹⁴C-L-leucine (230 mC/mmole) were obtained from New England Nuclear. Ecdysterone (isolated from plants) was obtained from Mann Research Laboratories. Synthetic α -ecdysone was a kind gift of Syntex Research. Schneider's medium and heat-inactivated fetal calf serum were obtained from Grand Island Biological Company.

Injection

Materials were injected into larvae by the method worked out by Mitchell and described by Boyd (2).

Preparation of Labelled Drosophila Soluble Proteins

Approximately 200 4-day old larvae are injected with ³H-leucine (4 mC/ml). About 2 hours later they are homogenized in a Duall grinder with 2 ml of pH 8.3 buffer (0.6 g/l Tris, 2.88 g/l glycine). The homogenate is centrifuged in the Servall SM-24 rotor for 1 hour at 15,000 rpm. The supernatant is decanted, care being taken to avoid decanting the fat pad, and proteins are precipitated by the addition of 4 ml of saturated ammonium sulfate. The precipitate is centrifuged for

15 minutes at 15,000 rpm in the SM-24 rotor and washed twice with 66% saturated ammonium sulfate in tris-glycine buffer. The precipitate is then redissolved in 1 ml of tris-glycine buffer and passed over a Sephadex G-25 column whose void volume has been previously determined by the use of dextran. The volume (approximately 2-4 ml) corresponding to that in which the dextran had emerged is collected and is lyophilized after an aliquot has been taken for counting.

Double Labelling

The ^3H -labelled proteins with their associated tris and glycine are redissolved in 0.1 to 0.2 ml distilled water. Ten μl of ^{14}C -leucine (50 $\mu\text{C}/\text{ml}$) are then added. Undissolved material is removed by centrifuging for 30 minutes at 11,000 rpm in a small conical tube placed in a rubber support in the Servall HB-4 swinging bucket rotor. An aliquot of the supernatant is then taken for counting, most of the remainder being injected into 300 to 500 animals at an age expected to be making granules (see Results).

Six hours later the animals are mixed with 10 g of frozen late 3rd instar larvae and granules are isolated according to the sucrose procedure described on pages 48-49. These granules are added to 1 ml of NCS solubilizer. They are dissolved either by being allowed to stand in the solubilizer overnight at room temperature, or by the addition of 0.2 ml of concentrated NH_3 and heating in the oven at 60° for 15 minutes. After the granules have dissolved, the solution is divided into two approximately equal portions (0.5 to 0.6 ml each), 5 ml of PPO/toluene fluor (5 g/l) is added to each, and each vial is counted in

the Beckman LS-200B scintillation counter.

Microscopic Visualization of the Granules

Fat bodies are dissected out in Drosophila Ringer's solution (4), then transferred to a microscope slide. This can most easily be accomplished by use of a 50 μ l disposable micropipette and a hand controlled suction apparatus. Excess Ringer's is removed from the fat bodies with a paper tissue and replaced with 1-2 drops of glycerol. The slides are then permitted to stand overnight or longer. (It has been found that stain does not penetrate the fat bodies well without the preliminary glycerol soaking.)

As much of the glycerol as possible is removed with a tissue and replaced with 1-2 drops of 0.25% Fast Green in 0.25 M Na_2HPO_4 which is well mixed with the remaining glycerol. The resulting pH is in the approximate range 7 to 8. This is due to acidity of the glycerol, presumably resulting from air oxidation of glycerol to glyceric acid. The slides are next allowed to stand at room temperature for 10 minutes; it is important that the amount of evaporation permitted be neither too great nor too small. Cover slips are added and the preparation squashed firmly with the thumb, then examined under the microscope.

Under these conditions the moderately stained blue to green granules stand out clearly against a pale blue to colorless ground cytoplasm. Nuclei stain light green, with nucleoli a fairly intense bright green.

The degree of granule development is indicated by subjective

assignment to one of five classes, from 0 (no granules) to ++++ (maximum development). Photographs illustrating these classes are included as Figure 3. Note that in terms of amount of granules the classes delineate an approximately logarithmic or power-law scale.

In Vitro Organ Culture

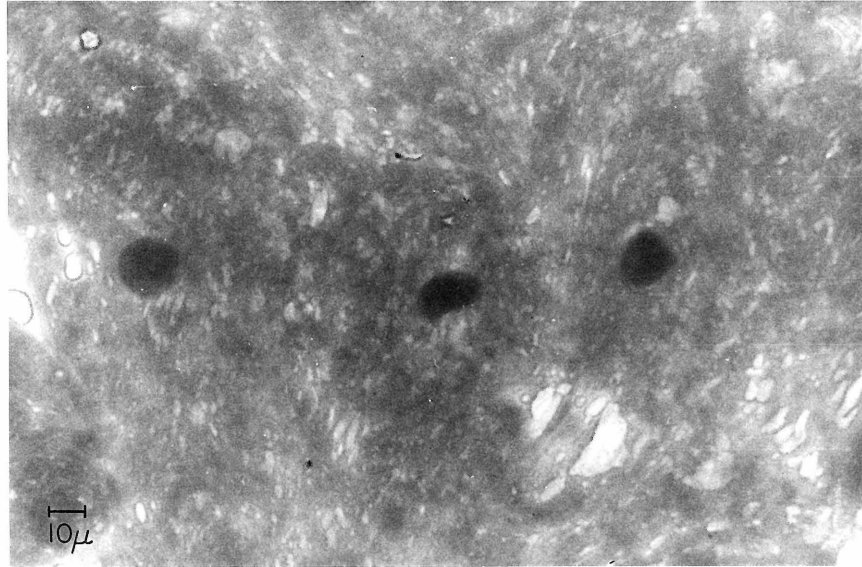
To each 100 ml of Schneider's medium are added 200,000 units of penicillin and 0.25 mg of streptomycin sulfate. Heat-inactivated fetal calf serum and/or protein, as needed, are added immediately before each experiment.

Since yeast grows extremely well in Schneider's medium and fungicides are frequently injurious to Drosophila tissues in culture, larvae for this purpose are grown axenically according to the procedure of Seecof (4). As development is slow under these conditions, the 70-hour old larvae routinely used as fat body donors are in the early portion of the 3rd instar.

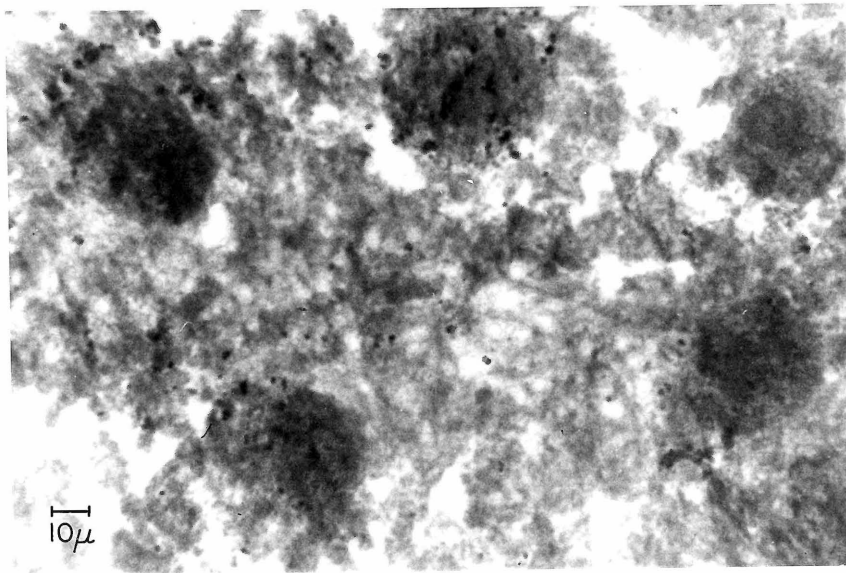
Under aseptic conditions, fat bodies are dissected out of donor larvae into the appropriate medium. In general, three fat bodies are transferred with 0.1 ml of medium to a cotton-stoppered 1/4 dram shell vial. In most cases 5 to 10 μ l of a hormone solution of appropriate concentration is added directly to the vial with a micropipette; this procedure leads to bubbling of air through the medium. In a few cases, discussed under Results, hormone was added to the bulk medium prior to dissection.

The vials, in a suitable beaker or rack to keep them upright, are transferred to a bell jar whose bottom has been covered with water.

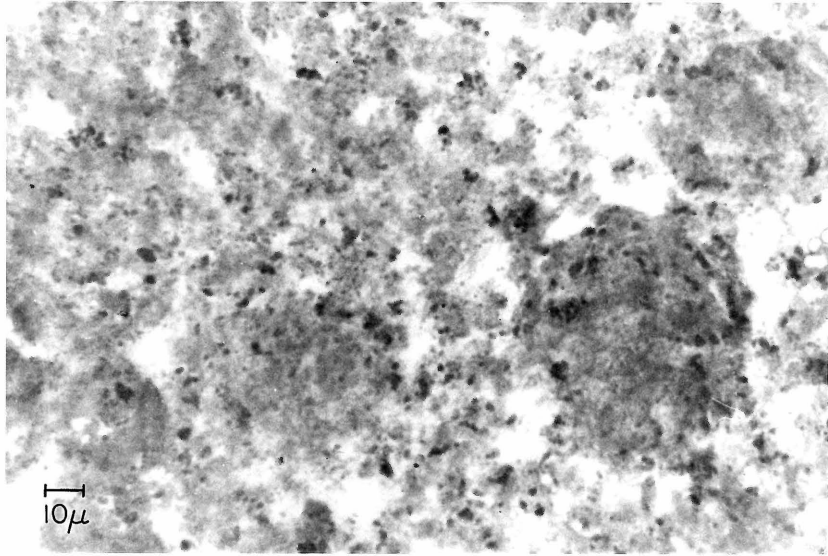
(a)



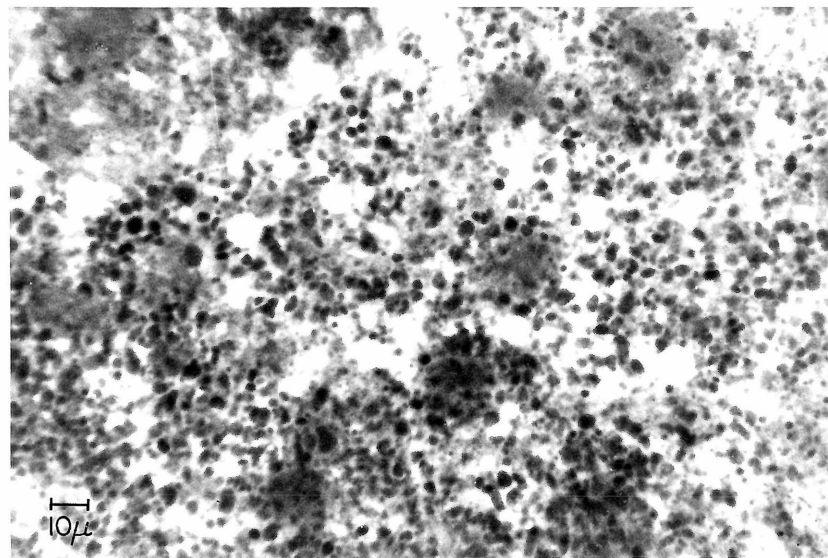
(b)



(c)



(d)



(e)

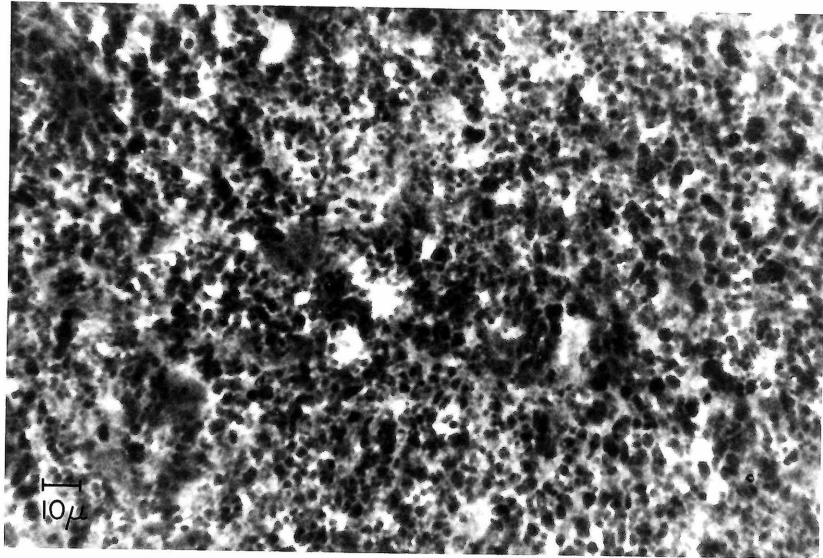


Figure 3. The stages of granule development.

(a) Stage 0; no granules. (b) Stage +; a few granules. (c) Stage ++; moderate number of granules (most typically somewhat more than illustrated). (d) Stage +++; many granules. (e) Stage ++++; very many granules, maximum development.

The bell jar is then placed in a 25° temperature-controlled room for incubation.

Vitality is checked by adding to fat bodies in one drop of medium about 2-4 μ l of 5% methylene blue, with mixing, followed by examination under the microscope 15-60 seconds later. Dead cells are heavily stained with blue color, while healthy cells are colorless or have only green nuclei.

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RESULTS

Isolation of Fat Bodies and Protein Granules

Two methods have been developed for the large-scale isolation of fat bodies from Drosophila larvae and of protein granules from the fat bodies. They differ only in that sucrose solutions are used extensively in one method, while their use is avoided in the other method. The second method is therefore more suitable for the preparation of granules which are to be examined for sugar content.

One hundred grams of sucrose (or 65 g of NaCl) are dissolved in sufficient Drosophila Ringer's solution to make 1 liter. Ten to eighty grams (wet weight) of larvae are washed through a power meat grinder (75-100 mesh, Quaker City Mill Company Model 4-E) with 150 to 600 ml of this solution. The effluent from the grinder is distributed into 250 ml glass centrifuge bottles which are filled to the top and centrifuged in the #259 swinging bucket rotor of the International Model PR-2 centrifuge at approximately 500 rpm (80 x g) for 15 minutes. The fat bodies are collected from the top with a pipette. If fat bodies remain stuck to the sloping neck of the bottle, the bottles are refilled, the fat bodies loosened by swirling, and the centrifugation repeated.

The material which floats on these solutions is 85-97% fat bodies. The most annoying contaminant--which is much worse with the NaCl solution--is cast larval skins. Any pupae which may have been among the larvae and escaped being ground will also float.

The fat bodies are homogenized in the 50 ml chamber of the Sorvall Omni-Mixer at top speed for 15 minutes. As the granules are

stable cooling is not necessary. This extreme degree of homogenization is required by the tendency of granules to embed themselves in masses of heavy fibrous material, possibly chromatin. The very extensive homogenization seems to chop up the fibers sufficiently to minimize this problem, though it is probable that there is still sufficient fibrous material to cause some loss of granules into the pellet in the final equilibrium sedimentation gradient.

Ten milliliters of homogenate are then layered on 20 ml of 48% (w/w) sucrose or 75% (v/v) glycerol. The interface is stirred sufficiently to eliminate a sharp boundary and the tubes centrifuged for 15 minutes at 10,000 rpm (16,000 x g) in the HB-4 swinging bucket rotor of the Servall RC-2 centrifuge.

The pellets are collected and combined in 2-3 ml of distilled water. If the material is heavily contaminated with cast larval skins or pupal cases it may be necessary to filter it through fine cheesecloth. The resuspended (or preferably rehomogenized) pellets are then layered on a 30 ml gradient from 0 to 10% (w/v) CsCl in 48% sucrose (or 23% to 34% (w/w) CsCl without sucrose) and centrifuged in the HB-4 rotor for 20 minutes at 10,000 rpm.

The material remaining in the gradient is primarily granules, most of which band around $\rho = 1.27$. Extensive rehomogenization followed by repetition of the equilibrium banding step leads to a narrower band and a purer preparation. Examination of such a preparation under the microscope shows it to be over 90% granules. There are some fragments of cast larval skin, and a few fibers or bits of cytoplasm adhere to the granules.

An alternative procedure for the preparation of granules has also been developed. This procedure gives a significantly less pure preparation, but is more convenient for handling small quantities of material.

In this procedure, fat bodies collected as before are permitted to sit in the refrigerator for 36-60 hours. During this time the fibrous material partially or largely disappears, while the granules are not affected. The fat bodies are then homogenized in a glass homogenizer (Duall or similar type). A clinical centrifuge tube is prepared with 4 ml of 66% sucrose on bottom; over this is layered 4 ml of 48% sucrose, and 2-3 ml of homogenate is layered on top. The tube is then centrifuged at the top speed of the International clinical centrifuge for one hour. Granules are collected from the interface between the two sucrose solutions. This material is approximately 60-80% granules.

Properties of the Granules

Solubility: The solubility properties of the granules are very simply summarized: they are completely insoluble in any reasonable biochemical solvent. A list of the solvents in which they failed to dissolve is included as Table 1. An attempt to dissolve the granules by succinylating the proteins with succinic anhydride (1) was also unsuccessful, whether or not the granules were first treated with 2% sodium dodecyl sulfate + 2% sodium deoxycholate to remove membrane lipids.

The granules could be partially "dissolved" by prolonged treatment with 6 M urea + 0.1 N NaOH, but the solubilized proteins were naturally degraded to peptides and amino acids by this treatment.

Solvents in Which the Protein Granules Did Not Dissolve

0.2 N HCl
6 M urea
2 M NaCl
0.2 N HCl + 6 M urea
0.2 N HCl + 6 M urea + 2 M NaCl
6 M urea + 2 M NaCl
6 M urea + 2 M LiCl
6 M urea + 1.5 M β -mercaptoethanol
6 M urea + 2% sodium dodecyl sulfate
6 M urea + 2% sodium dodecyl sulfate + 0.2 N HCl
8 M urea + 3% NH_3 + 2% Triton X-100
2% sodium deoxycholate
6 M guanidinium chloride *
66% (v/v) acetic acid
formic acid **
perflouracetone **

* in some cases this was adjusted to pH 12 with 20% KOH

** these materials were added in anhydrous form to wet pallets of granules

In general, granules were mixed with the indicated solvent and left overnight at room temperature; the granules were examined visually to determine whether they had dissolved. In case of doubt as to whether they had partially dissolved, the granules were pelleted and the supernatant examined for material absorbing at 280 nm and for TCA precipitable material.

Other properties: An amino acid analysis of granules prepared by the alternate method described on page 50 is included as Table 2.

The Burton diphenylamine test (2) for DNA and the orcinol test (3) for RNA were both completely negative.

An analysis for phosphorus (4) showed quite minimal amounts, approximately 1 gram-atom of phosphorus per 100,000 grams of protein.

Origin of the Protein in the Granules

Two lines of evidence prove that in Drosophila, as in Calpodes, essentially all the protein in the granules is preformed protein sequestered from the hemolymph, rather than protein synthesized in situ at the time of granule formation.

In the first series of experiments, animals were injected at the approximate time they were expected to be making granules with a mixture of ^{14}C -labelled leucine (as the free amino acid) and ^3H -labelled Drosophila soluble protein, and the resulting granules were collected and counted. Detailed experimental procedures are given on pages 39-41.

Experiment 1 was done with larvae hatched from eggs collected 101 to 107 hours before injection. Development of these larvae, however, was unusually rapid, and about one quarter of the animals had pupated by the time of sacrifice. Therefore, most of these animals had completed granule formation by the time they were injected. The counts recovered were from animals developing more slowly than most, and represent only the last proteins incorporated.

TABLE 2

Amino Acid Analysis of the Granules	
Amino Acid	Mole %
Aspartate	10.5
Threonine	5.8
Serine	6.0
Glutamate	11.5
Proline	5.3
Glycine	8.6
Alanine	8.7
Cysteine	trace
Valine	6.9
Methionine	2.4
Isoleucine	5.1
Leucine	8.5
Tyrosine	3.4
Phenylalanine	4.4
Histidine	*
Lysine	7.4
Arginine	5.3

* The histidine peak was not well separated from that due to an amino sugar, presumably glucosamine from the contaminating chitin.

Experiment 2 was done with larvae hatched from eggs collected 98 to 100 hours before injection. Ten hours later about 15-20% had pupated, suggesting a nominal time of pupation about 12 hours after injection. The animals should therefore have been on the average about half way through the process of granule formation.

The results for both experiments are given in Table 3. These figures are given as raw cpm, unadjusted either for background or for varying counting efficiencies as revealed by external standardization. The number of cpm in the aliquot of the injected solution from which the $^3\text{H}/^{14}\text{C}$ ratio was determined was sufficiently great to render the background negligible. Since in both experiments the counting efficiency for the injected solution was lower than that for the granules, adjustment for this factor could only increase the support for the conclusions drawn below.

It is quite clear from both experiments that ^3H -labelled proteins are incorporated into the granules to a very much greater degree than are ^{14}C -labelled amino acids. In fact experiment 2 indicated that there is essentially no incorporation of free amino acids into the protein of the granules.

The calculated $^3\text{H}/^{14}\text{C}$ for the injected solution was 0.695; if the injected amino acids had been incorporated into protein, and this protein had been incorporated into granules with the same efficiency as the injected protein, we would have expected 427 and 489 ^{14}C cpm in the two samples. The maximum number of ^{14}C cpm which the statistics will permit is approximately 2.

TABLE 3

RESULTS OF DOUBLE-LABELLING EXPERIMENTS

Experiment 1				
Injected $^3\text{H}/^{14}\text{C} = 10.5$ (Ext. Std. = 0.834)				
		^3H	^{14}C	External Standard
Granules	Vial 1	265.0 ± 7.9	12.6 ± 1.9	0.881
	Vial 2	179.0 ± 8.9	12.4 ± 1.9	0.891
Blank		19.0 ± 2.8	13.3 ± 2.0	1.413
Experiment 2				
Injected $^3\text{H}/^{14}\text{C} = 0.695$ (Ext. Std. = 0.758)				
		^3H	^{14}C	External Standard
Granules	Vial 1	315.3 ± 4.7	14.5 ± 1.0	1.206
	Vial 2	358.0 ± 5.4	14.4 ± 1.0	0.950
Blank		19.1 ± 1.0	15.3 ± 1.1	1.112

^3H and ^{14}C are cpm ± 1 standard deviation

The yield of ^{14}C in this experiment is in fact so low as to be almost embarrassing. The labelled amino acid must surely be incorporated into protein, some of which would be expected to enter the hemolymph and be incorporated into the granules. Quantitatively, however, the hemolymph proteins probably represent no more than 5% of the total protein of the insect; if the proteins of the insect were in radioactive equilibrium at the time of granule formation, and if 60% of the hemolymph proteins were incorporated into granules (as seems to be the case in Calpodes), then only about 3% of the injected amino acid would end up in the granules.

Furthermore, the insect proteins are not in radioactive equilibrium. In Calliphora, Martin et al. (5) found that very little radioactivity appeared in the hemolymph proteins in the first 90 minutes after injection. This lag is important since the animals in experiment 2 completed granule formation on the average four hours after injection, and may have finished taking up the protein which was to be incorporated significantly earlier. In addition, Martin et al. found that during the last half of the final larval instar the specific radioactivity of the hemolymph proteins--at any time following injection--remained significantly lower than that of any other tissue examined. The virtual absence of incorporation of labelled amino acid into granules via newly synthesized hemolymph proteins is therefore reasonable.

The second line of evidence demonstrating that the proteins in the granules are not synthesized at the time of granule formation rests on an experiment in which cycloheximide was injected shortly before the beginning of granule formation. About 20 hours before puparium

formation--therefore about four hours before the first visible appearance of the granules--larvae were injected with 2 nmoles of cycloheximide. This amount of cycloheximide is known to effectively inhibit protein synthesis (6). Sixteen hours later six larvae were dissected and their fat bodies examined for protein granules. In all six fat bodies the granules were completely normal in size, number, and appearance. This is clear proof that not only are essentially all the proteins in the granules preformed, but that virtually no synthesis of any protein (unless one whose synthesis is exceptionally resistant to cycloheximide) need take place at the time of granule formation.

Time at Which Granules Appear

In Drosophila, as in other animals, even though larvae are raised under apparently identical conditions they show significant variability in the time at which they reach a given developmental stage such as pupation. This variability is accentuated by the fact that Drosophila females retain their eggs for varying lengths of time, so that some have reached different stages of embryological development at the time they are laid.

As granule formation occurs during the day before pupation, it would be expected that the time at which it occurs would be more stably related to the time of pupation or puparium formation (pupariation) than to the age from hatching. It is, however, obviously impossible to know the time of pupariation for a larva which has already been dissected or otherwise experimentally manipulated. It is accordingly necessary to have a population of larvae whose development is as synchronous

as possible, so that the time of pupariation for an individual larva can be at least roughly estimated from the mean pupariation time for the population.

To this end, eggs are collected over a two hour period. About 20 hours later, as the eggs are beginning to hatch, unhatched eggs are picked up on a brush and transferred to moist filter paper. A maximum of 45 minutes is allotted to this. After waiting 30 minutes, larvae which have hatched during this period are transferred to a petri dish of Drosophila food. No more than 150 larvae are grown in any dish. This transfer is also allotted a maximum of 45 minutes, so that all larvae in the population have hatched within a two hour period.

Under these conditions the times at which individual larvae pupariate show a Gaussian distribution about the population mean, with a standard deviation of approximately ± 2 hours. The population means also seem to show a Gaussian distribution about their average value, with a similar standard deviation. However, the mean pupariation time for a given population is directly determinable.

At various times during the day before pupation larvae were dissected and the fat bodies stained for granules. In Figure 4, the degree of development of the granules in the fat body of each larva is correlated with the number of hours between the time at which it was sacrificed and the average time at which larvae in that population pupariated. In addition to the three specific experiments whose results are combined in Figure 4, granules--or their absence--were frequently observed in the course of experiments performed for other reasons. All observations were consistent with the conclusion that granules are

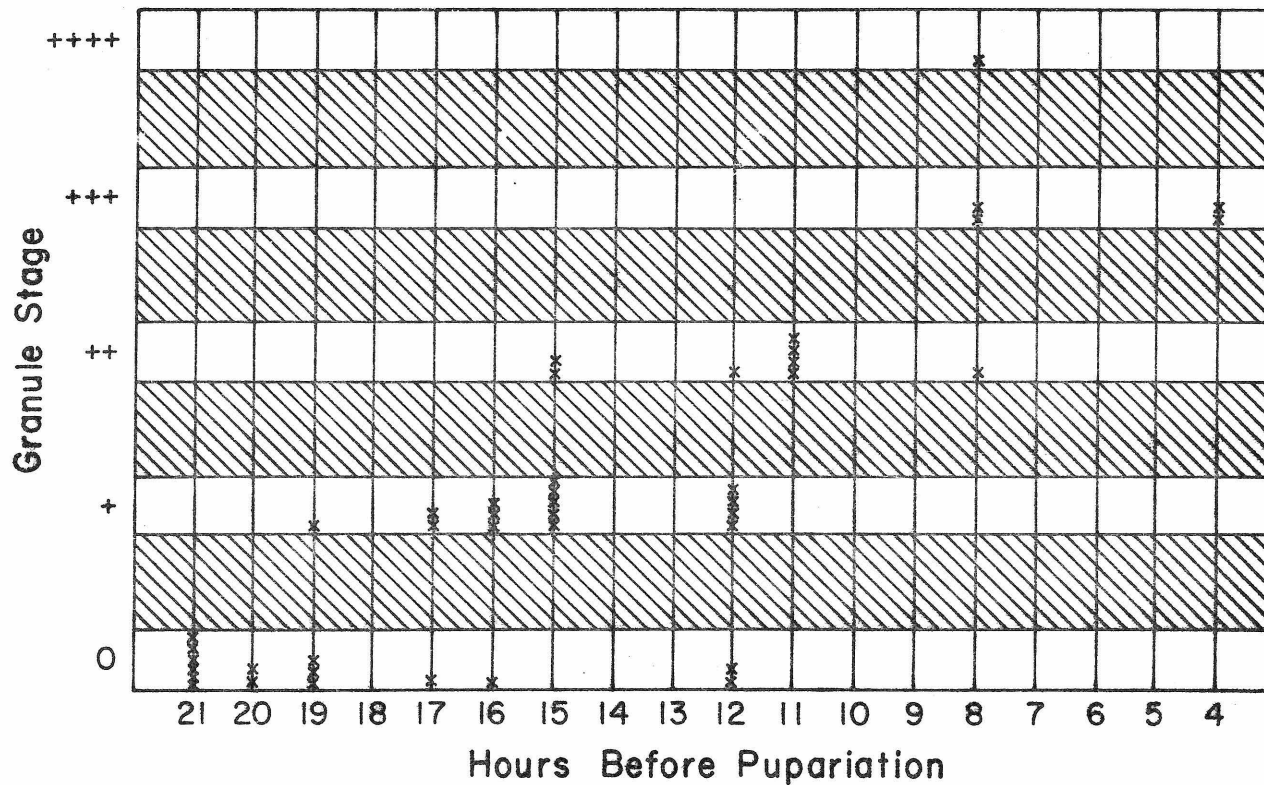


Figure 4. Correlation of stage of granule development in individual larvae with mean time of pupariation for the population from which they were taken. Each x represents one larva. Results of three separate experiments are combined in this figure.

first visible 17 ± 2 hours before pupariation and reach their maximum development within eight to nine hours. The two larvae which showed no granules 12 hours before the mean pupariation time for their population were undoubtedly slow developers; one of them was noted at the time of dissection to appear immature for its age.

The uncertainty in the time at which granules appear is the same as the uncertainty in the time of pupariation for individual larvae in a population. There is no evidence for variability in time of granule formation relative to time of pupariation.

Some larvae show a maximum granule development of +++, while others never get beyond the ++ stage. This apparent difference has not been investigated, though it should be noted that in Calpodes females have 50% more granules than males (7).

Ligation Experiments

In ligation experiments, a hair or fine wire is tied around the middle of the animal so that ecdysone produced in the front half of the animal does not reach the rear half; this half therefore does not pupate. Experiments of this sort were the key to Collins' demonstration that ecdysone is the inducer of the protein granules in Calpodes (3).

Ligation experiments are in principal equally useful in Drosophila. Technically, however, they are extremely difficult due to the small size of the larvae. If the wire is too tight, the animal dies; if it is too loose, pupation occurs normally in both halves. I ligated over 200 third instar larvae--4 of these ligations appeared successful.

In three of these animals the fat body of the rear half was examined for granules. In each case granules were present in normal degree. It is possible, however, that even though the rear half did not pupate normally the ligation was nevertheless loose enough to permit some passage of hormone.

Induction of Granules by Ecdysterone In Vivo

One key piece of evidence in the argument that ecdysone induces granules would be the demonstration that injection of ecdysterone can cause the formation of granules prior to their normal time of appearance. This is the case.

When early third instar larvae (approximately 44 hours before pupariation) are injected with 160-200 ng of ecdysterone in Ringer's solution + 3% ethanol, granules are first visible approximately eight hours later and reach their maximum development about six hours after this. The granules produced in this way are often larger but less numerous than those occurring naturally, as is illustrated in Figure 5. Injection of ethanol-Ringer's solution does not produce granules.

The maximum development of granules produced by injection of ecdysterone is somewhat difficult to quantitate, not only because the distribution of granule sizes differs from normal, but also because the degree of development varies both between animals and between different parts of the fat body of the same animal. In general, the maximum development observed in any one animal lies in the range from ++ to ++++. It is not uncommon to find portions of the fat body which have no granules at all, often immediately adjacent to apparently identical

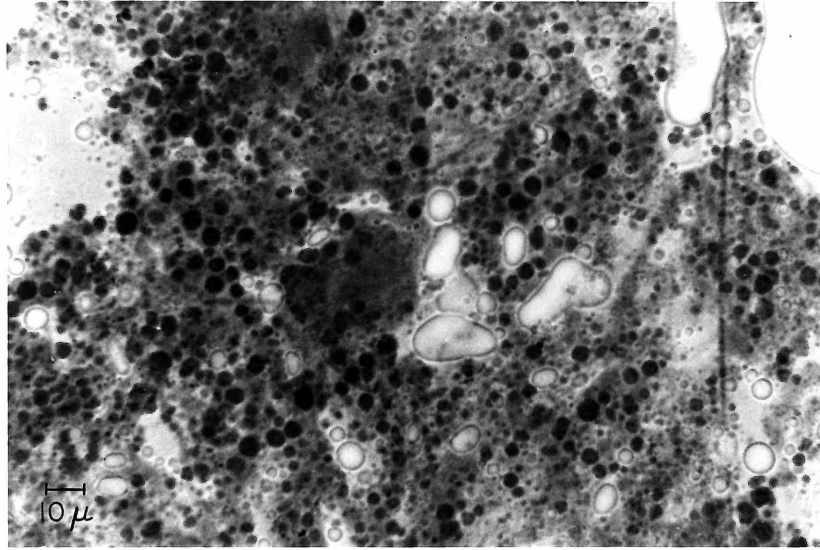


Figure 5. Granules induced by ecdysterone.
Note large size.

portions where they are numerous.

Injection of smaller amounts of ecdysterone does not affect the type or degree of response obtained, but does reduce the proportion of animals showing this response. When 40 ng/animal is injected, about 75% of the larvae form granules precociously; when 20 ng/animal is injected, the response is very sporadic. For comparison, Karlson & Hanser (9) have found that the minimum quantity of ecdysone necessary to produce pupation in the rear half of ligated Drosophila larvae is 20 ng (2 Calliphora units).

That the period of eight hours between injection of hormone and the first visible appearance of granules is a true lag or induction period, not dependent on the age of the animals, is supported by the observation that injection of hormone eight hours before the granules are naturally due to appear does not result in any acceleration of their appearance.

If 2 nmoles of cycloheximide were injected concurrently with the hormone, induction of the granules was prevented. Granules were still not present two days later, when uninjected controls had pupated, suggesting that the natural induction process had also been blocked. However, the cycloheximide-injected animals (whether or not also injected with hormone) pupated one or two days later than the controls, so it is possible that granules appeared during this period.

Since the experiments reported on pages 56-57 established that no protein need be made at the time of granule formation, these results suggest that ecdysone induces the synthesis of a specific protein which is probably catalytic in function, inasmuch as enough of it to make all

the granules is already present when the first granules appear.

Induction of Apolysis by Ecdysterone

The term apolysis was coined by Jenkin and Hinton (10) for the process by which an arthropod forms a new cuticle separate from the old one. Ecdysis is then the process by which the old cuticle is shed, completing the molting process.

Beck and Shane (11) applied this term to the phenomenon which they observed on injecting ecdysterone into diapausing prepupae of the European corn borer, Ostrina nubilalis. In this case a more or less complete cuticle is formed under the old one, which is never shed. The new cuticle may show either larval or pupal characteristics to varying degrees, but true pupal development is not initiated and the animals die within a few days.

I now report that injection of ecdysterone into early third instar larvae of Drosophila melanogaster produces a similar phenomenon, which generally becomes apparent 20-30 hours after injection. While the development of the syndrome is quite variable, the general characteristic is the appearance of a thin, new cuticle underneath, but separated from, the old one. This cuticle is always incomplete; often it is present only in the rear half of the animal. In many cases the tissues undergo a rearrangement resembling that following the prepupal molt. As the animals are typically flaccid and consequently hyperextended, this produces a clear space at the head which is the most grossly obvious symptom of the syndrome. In a very few cases imaginal discs evert and pupal development appears to be initiated. Hist-

olysis of larval tissues, however, is generally less than would be expected in normal prepupae or pupae. Affected animals generally die before development is highly advanced.

In order to determine the dose necessary to achieve this result, groups of 40 larvae were each injected with one of the following amounts of ecdysterone: 0, 10, 40, or 160 ng/animal. The number of adults eclosing was determined as a percentage of the larvae injected, and the results plotted on a semilogarithmic scale as Figure 6. In each group 3-5 animals pupated but did not eclose. These are treated as non-survivors; treatment as survivors would not materially alter the conclusions.

The results for the three ecdysterone treated groups lie on a straight line passing through the origin. This line implies a logarithmic dose-response relationship with an LD_{50} slightly over 50 ng/animal. However, this line would also imply 100% survival in the controls, which is not the case in these experiments. In the course of numerous injection experiments it has been found that survival averages around 95%, but is quite variable; a value as low as 80% would not be extremely unusual. It is not clear whether or not it is appropriate to take this value as the baseline for the other groups in this experiment. If it is, then 10 ng/animal appears as a threshold value, and the true LD_{50} (40% overall survival) is 70 ng/animal.

Similar experiments were carried out using a saturated solution of α -ecdysone in 12% ethanol-Ringer's. The results were generally equivalent except that the larvae were contracted rather than hyperextended.

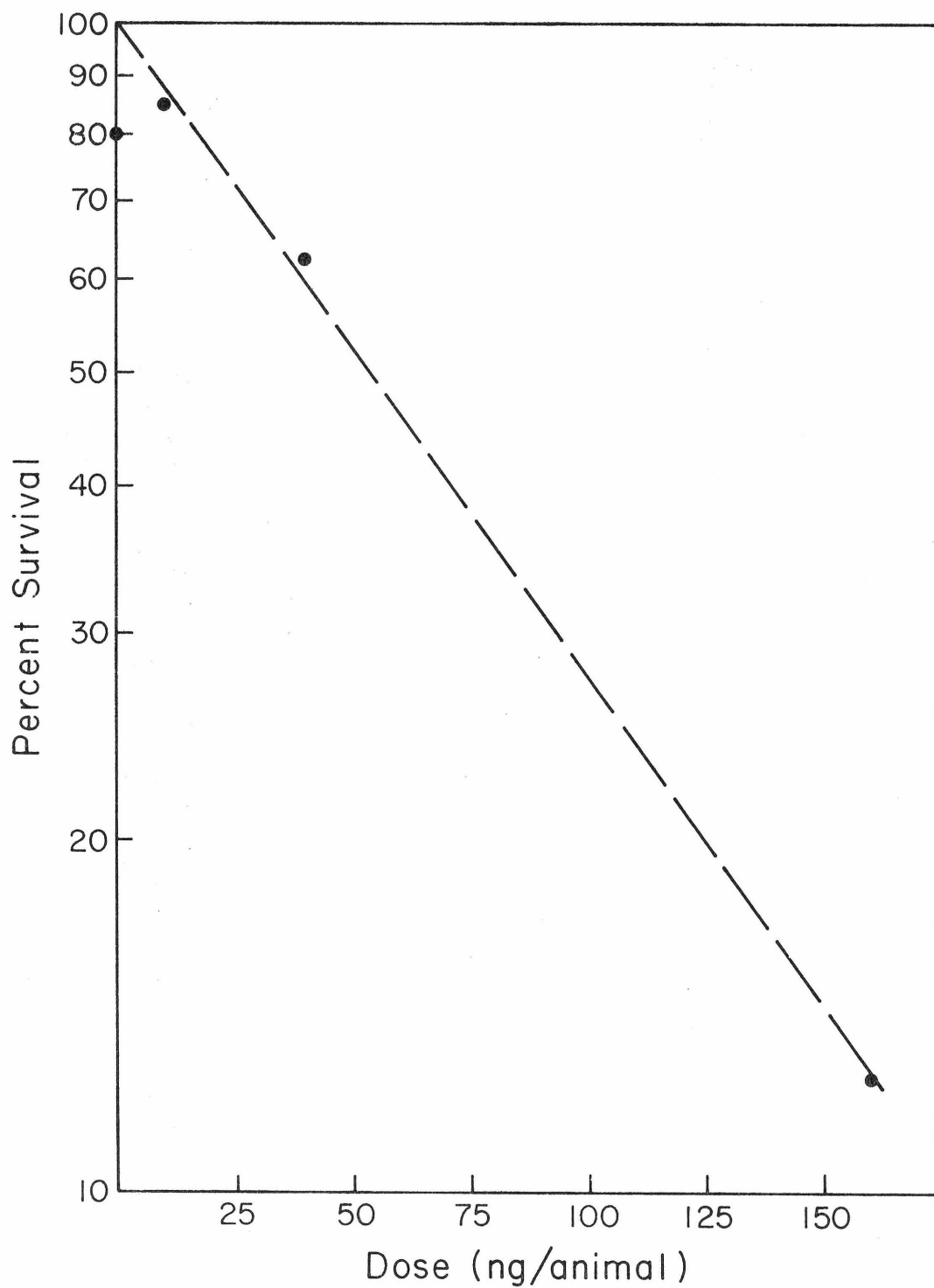


Figure 6. Percentage of larvae injected with the indicated dose of ecdysterone which survived to adulthood.

Induction of Granules In Vitro: Media with Serum

The most precise experiments on hormonal control mechanisms can be expected to be done in vitro. In vitro there are no confounding variations in the physiological states of the animals and no secondary changes in conditions due to effects of the hormone on other organs. The external conditions are precisely known and can be independently varied.

I accordingly began a series of attempts at inducing granules in organ culture in vitro. Schneider's medium, although developed to permit differentiation of Drosophila eye-antennal discs in culture (12), did not permit adequate survival of fat bodies. I accordingly added 15% heat-inactivated fetal calf serum, as Seecof has done for long-term maintenance of opened whole larvae and for differentiation of embryonic cells (13). In this medium survival, as judged by decolorization of methylene blue, is excellent for at least the three days which was the maximum period to which my experiments extended.

Results of these experiments are somewhat ambiguous and difficult to interpret, since in control cultures without added hormone granules are observed in about half the cases. There is no obvious explanation for this variability, though it is notable that replicate cultures from any given experiment usually agree with each other.

However, when ecdysterone--in quantities as small as 20 $\mu\text{g}/\text{ml}$ --is added to the cultures, granules form consistently. These granules resemble those formed in response to injection of hormone in vivo in being both frequently larger and less numerous than those naturally

observed, and also in their patchy distribution in the fat body. Addition of more hormone, up to 1.25 mg/ml, does not affect these results in any way.

When ovalbumin at concentrations in excess of 50 mg/ml is added to this medium, granules form consistently in the absence of added hormone. Lower concentrations of this protein are without effect. The granules formed are rather uniform in size, somewhat smaller than the average naturally appearing granule. Addition of hormone at concentrations up to 1.25 mg/ml did not cause the production of larger granules.

In order to determine the period required for the appearance of granules in vitro, ecdysterone at a concentration of 0.25 mg/ml was added to medium without added protein and replicate cultures examined for granules at 9, 24, 33, 48, 57, and 72 hours. There were no granules at 9 or 24 hours; at 33 hours the presence of granules was quite obvious, and at 48 hours and all later times they were quite large and well developed.

During these experiments some cells were noted to have numerous regularly spaced, small, darkly staining granules. Many of these cells have pycnotic nuclei and are presumably dead as a result of mechanical injury during dissection. (Vital staining with methylene blue revealed patches of dead cells after as little as nine hours of incubation.)

Induction of Granules In Vitro: Media without Serum

As I suspected that the variable results in the cultures without

added hormone resulted from ecdysone-like factors in the serum, I turned to the use of a more defined medium. I found that I obtained very good survival if to Schneider's medium I added protein at concentrations of at least 20 mg/ml (I regularly used 30 mg/ml). While I regularly used either human or bovine serum albumin as the protein, experience suggests that any protein not obviously unsuitable would give satisfactory results.

I was initially under the impression that granules did not form in this medium unless hormone was added. I later found, however, that addition of hormone is not necessary if air is bubbled through the cultures as they are being set up--as is done in the process of adding hormone by pipette. Granules did not form in one culture in which hormone was added to the bulk medium prior to dissection, so that no air was bubbled through it. However, all attempts to replicate this result were unsuccessful, even when the culture was deaerated by bubbling nitrogen through it.

The granules which form without addition of hormone resemble those which form in the serum-containing medium with added ovalbumin, while hormone induces the formation of granules resembling those induced by hormone under other circumstances. Addition of hormone induces formation of granules during the first day of culture, while in its absence they do not form until the second day. There is therefore a hormone effect in these cultures even though hormone is not essential for the appearance of granules.

At one point in these experiments I tried using as my added

protein fetuin, a sialoprotein isolated from fetal calf serum. To my surprise, this completely prevented granule formation, even in the presence of serum and 1.25 mg/ml of hormone. With fetuin present at 20 mg/ml, this is a protein/hormone ratio of 8. This ratio seems much too low for the results to be accounted for by binding of hormone to protein. I have no simple explanation to offer for this phenomenon.

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DISCUSSION

I stated earlier that this thesis would be devoted primarily to the elucidation of two questions: are the proteins in the granules of Drosophila melanogaster, like those of Calpodes, primarily sequestered hemolymph proteins, and what is the role of ecdysone in the induction of the Drosophila granules?

The answer to the first question is unequivocal. In Drosophila, the proteins in the granules are not just primarily, but almost exclusively sequestered from the hemolymph. Proteins synthesized in situ at the time of granule formation constitute a negligible proportion of the granules if they are incorporated at all. Discounting the possibility of a protein whose synthesis is exceptionally resistant to cycloheximide, it is quite clear that proteins synthesized at the time of granule formation play no essential role in the process.

It appears functionally reasonable that this should be the case. The process of puparium formation, followed by the prepupal and pupal molts, must make a severe metabolic demand on the animals. This is accentuated by the fact that they cease feeding six hours or more before pupariation. The synthesis of the rather large quantities of protein contained in the granules must also be a metabolic drain of some importance, especially if it were concentrated in one organ and within the space of eight hours. By separating synthesis of the protein from the process of granule formation it becomes possible to spread the synthetic process over a much longer period, one in which demands on the animal may be much less severe.

The answer to the second question is much less clear. There is

convincing evidence that ecdysone can induce the formation of granules. The precocious induction of granules by injected ecdysterone is the most striking example of this. The accelerated production of granules in vitro in the presence of ecdysterone, and the greater consistency of granule formation when it is added to media containing serum are further evidence. These experiments in vitro also demonstrate that the effect of the hormone is directly on the fat body, rather than being secondary to an effect on some other organ. The concept of ecdysone as an effective inducer had, of course, already received strong support from the experiments of Butterworth et al. (1) showing that implantation of ring glands was necessary for production of granules in fat bodies incubated in adult male hosts.

Nevertheless, there is also convincing evidence that ecdysone is not necessary (in Drosophila) for granule formation. Again, this had been shown by the experiments of Butterworth and Bodenstein (2) on implantation of fat bodies into adult female hosts. It is true that they interpreted their results in terms of a hormone from the ovary, and possibly the corpus allatum, which would in this respect mimic the action of ecdysone. However, the results presented in this thesis show that this conclusion is by no means compulsory.

The results obtained when fat bodies are incubated in vitro in Schneider's medium plus albumin indicate strongly that under appropriate conditions they will make granules spontaneously, with no necessity for a hormonal trigger of any kind. One caveat, however, must be added to this conclusion. Schneider's medium is not strictly defined; it contains 2 mg/ml of yeast hydrolyzate. It remains conceivable, in

view of the isolation of ecdysones from plants, that this hydrolyzate contains ecdysone-like substances. However, it seems unlikely that they could be present in the necessary concentrations. Both in vivo and in vitro, concentrations (in hemolymph or medium) less than 20 $\mu\text{g/ml}$ have tended to give erratic results; in order for the ecdysone-like substance to reach this concentration in the medium it would have to represent 1% of the weight of the hydrolyzate. This seems extremely unlikely.

The results of the ligation experiments, while slightly questionable because of the technical difficulties, also favor the conclusion that ecdysone is not necessary for granule formation.

The experimental results, then, indicate that ecdysone is sufficient but not necessary for granule induction. But what role does it play in natural induction of the granules? There is no conclusive evidence on this point, but one consideration argues that ecdysone is probably not involved in the natural appearance of the granules. This is the matter of timing.

The granules naturally appear 17 hours before pupariation. The experiments with injection of hormone indicate that there is a lag period of eight hours between the action of the hormone and the first visible appearance of the granules. If, as seems likely, this would also be true for naturally secreted ecdysone then the hormone must make its first appearance at 25 hours before pupariation. Yet ligation at any time up to four hours before pupariation effectively interferes with pupation in the posterior half (3); this is naturally interpreted to imply that hormone is first secreted at this time.

These two sets of observations are not wholly irreconcilable. It is possible to assume, for example, that the fat body is sensitive to lower concentrations of ecdysone than is the epidermis, and that small amounts of hormone are secreted by the prothoracic gland throughout the last day of larval life, but high concentrations are produced only during the last four hours. However, neither injection experiments nor in vitro incubations suggest any difference between the amount of hormone required to induce granules and that required to induce apolysis or pupariation.

If we are then to reject ecdysone as the natural inducer of granule formation, is it possible to suggest how this process is actually controlled? I believe that the evidence does in fact suggest a plausible hypothesis: that the appearance of granules is prevented by juvenile hormone (JH); they appear spontaneously as soon as the JH titer falls below a certain minimum.

This hypothesis is obviously in accord with the results of the in vitro incubation experiments. As nothing quantitative is known about the rate of decline of the JH titer during the last larval instar, the hypothesis also cannot be contradicted on this ground. Even if data of this sort were available, the possible existence of a lag or induction period between activation and the first visible appearance of the granules would prevent this from being considered a crucial test.

The necessary data do not exist to ascertain whether this hypothesis can explain the results of Butterworth and his coworkers. It is certain that JH exists in adults, since in insects generally it is necessary for egg maturation in the female and for development of the

accessory sex glands in the male (see references (4) for reviews). While there does not seem to have been any quantitative work done on JH titers in adult Diptera, Gilbert and Schneiderman (5), in an extensive survey of Lepidoptera, found that the male had higher titers than the female--in one extreme case 40 times as much--in 15 out of 17 species examined. My hypothesis would imply that this is also the case in Drosophila.

My hypothesis also implies that the concentration of JH is higher in the fes mutant than in normal females. This has been a subject of controversy for some time (6,7). The corpus allatum is grossly hypertrophied in mated females of this genotype--a condition which can be normalized by the implantation of wild-type ovaries--and Doane interprets this as indicating overproduction of JH due to lack of an inhibitory influence from the ovary (6). King et al. (7), however, note that the gland seems to be storing abnormal quantities of hormone, and propose that what is lacking is an ovarian product necessary for JH secretion. According to this interpretation, the JH titer in the hemolymph is abnormally low. I would suggest, however, that the buildup to a high JH titer might itself inhibit secretion.

It is important to emphasize that these conclusions and the resulting hypothesis are intended to apply only to Drosophila melanogaster. There is no reason whatsoever to question the validity of Collins' (8) conclusions for Calpodes ethlius, and it is quite possible that the mechanism elucidated for Calpodes is the typical one while that of D. melanogaster is atypical.

Drosophila melanogaster was chosen for genetic work largely because of its exceptionally short life cycle. This shortening is particularly extreme in the last larval instar. This instar lasts only two days in D. melanogaster, as compared to eight days in Calpodes ethlius and eleven days in Calliphora stygia. The time between ecdysone secretion and pupariation is shortened still more--four hours in D. melanogaster but thirty six in Calpodes. Thirty six hours is sufficient time to make granules; four hours is not. It seems probable that in the course of its evolutionary adaptation to evanescent sources of food D. melanogaster has had to abandon the primitive mechanism of triggering granule production by ecdysone in favor of a mechanism which will permit an earlier start in this process.

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