METABOLISM OF PROTEIN AND NUCLEIC ACIDS DURING THE
METAMORPHOSIS OF TENEBRIO MOLITOR L.

by

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**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>DNA</td>
<td>Deoxy ribose nucleic acid</td>
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<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>sRNA</td>
<td>Soluble (transfer) RNA</td>
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<tr>
<td>TCA</td>
<td>Tri-chloro acetic acid</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric acid</td>
</tr>
<tr>
<td>PP</td>
<td>Pyrophosphate</td>
</tr>
<tr>
<td>P</td>
<td>Orthophosphate</td>
</tr>
<tr>
<td>PRPP</td>
<td>5-phosphoribosyl - 1 - pyrophosphate</td>
</tr>
<tr>
<td>TPN</td>
<td>Triphosphopyridine nucleotide (oxidized).</td>
</tr>
<tr>
<td>TPNH</td>
<td>Triphosphopyridine nucleotide (reduced)</td>
</tr>
<tr>
<td>Glucose-U-C(^{14})</td>
<td>Glucose uniformly labelled with C(^{14})</td>
</tr>
<tr>
<td>DOPA</td>
<td>3,4 Dihydroxyphenylalanine</td>
</tr>
<tr>
<td>E</td>
<td>Efficiency for incorporation of glycine into protein</td>
</tr>
<tr>
<td>Eu</td>
<td>Efficiency for incorporation of uridine into RNA</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>A-T</td>
<td>Base pair of adenine and thymine</td>
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<tr>
<td>G-C</td>
<td>Base pair of guanine and cytosine</td>
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SUMMARY
GENERAL INTRODUCTION

THE CONTROL OF GROWTH AND DEVELOPMENT IN INSECTS

In holometabolic insects development proceeds through several larval and a pupal instar. The larva differentiates into the adult via the pupa. The transition from the fully grown larva to the adult organism is called metamorphosis. The change from each instar to the next involves molting, during which the old chitinous exoskeleton is shed and a new one formed.

Post embryonic growth of higher organisms is under hormonal control. Hormonal control in vertebrates and higher plants has been studied extensively. Increasing attention, however, is at present being paid to invertebrates, particularly the arthropods.

Since insects have a rigid and impermeable exoskeleton, growth is in successive steps. In order to increase in size the larva molts periodically. The pupal stage is characterized by breakdown of the larval tissues. This breakdown differs in extent from species to species and can be partial or complete. The breakdown of tissues takes place in an orderly sequence and the last tissue to disappear is the fat body. Histogenesis follows histolysis. Imaginal structures are formed from the histoblast. Cells, tissues and organs are reconstructed from larval elements in situ.

Moulting and metamorphosis are under hormonal control. The physiology of the latter process has been studied extensively, especially the hormonal control of metamorphosis. The neuroendocrine basis of this process has been demonstrated and some of the active principles
involved have been chemically characterized. However, the biochemical events that occur during this phase of insect life remain unknown.

The process of moultling is a distinguishing feature of insect growth and development. Growth is only possible when the cuticle, which is attached to the epidermis, detaches itself from the latter. The epidermis then grows by cell division, by cell enlargement or by both processes and secretes a new extensible folded cuticle. When the new cuticle is almost complete, specific enzymes produced and released by the epidermal cells, digest the inner layers of the old cuticle, and the digested material is absorbed by the epidermal cells. The latter now wax and waterproof the new cuticle and the old cuticle is shed. An insect with a rigid body fills itself with air immediately after the moult when the cuticle is soft. The enlarged rigid skin now provides room for the growth of the internal organs. In soft-bodied insects the skin remains wrinkled in the beginning and stretches gradually. In many insects growth in cell size occurs between ecldyses, and cell division occurs mostly at the time of moultling in both the epidermis and the internal organs. In this respect insects differ from mammals in which growth of the immature is continuous.

Three hormones control growth and development of insects. Two of these, the brain hormone, produced by the secretory cells of the brain and ecdysone, which is produced in the prothoracic glands, are responsible for the periodic cell multiplication and moultling. The brain hormone stimulates the prothoracic glands to secrete ecdysone, which is the insect growth hormone. The latter stimulates the epidermal cells to divide, to deposit new cuticle and finally to moult. The effect of ecdysone can be demonstrated on isolated pupal abdomens.
which can "live" as such for a year. Moulting will occur in isolated abdomens after they are injected with a few micrograms of ecdysone.

The third hormone is the juvenile hormone, produced in the corpora allata, which are located near the insect brain. In moulting larvae, when the juvenile hormone is present in a sufficient concentration, the larvae do not differentiate and retain larval characteristics. On the other hand, when the hormone level is low, the larvae pupate. In the absence of hormone the pupae moult and become adults. When larvae are stimulated to grow and moult by ecdysone, the presence of a high concentration of juvenile hormone before the moult will maintain the insect in the larval stage.

**THE BRAIN HORMONE(S)**

In 1922 Kopeck (2) reported the results of biological experiments, which indicated the role of the cerebral ganglia as endocrine glands. The first extracts with brain hormone activity were prepared in 1958 by Kobayashi and Kirimura (3). They extracted an oily material from 8,500 silkworm brains. Injection of 0.1 mg of this extract into brainless silkworm pupae caused them to moult. These workers claimed that the active principle was a lipid. Later Ichikawa and Ishizaki (4,5) obtained a water soluble extract with brain hormone activity and this appeared to be due to a polypeptide. In 1962, Kirimura et al reported the isolation of active crystalline substance which they identified as cholesterol (6,7). Karlson, however, (8) doubted that cholesterol is the brain hormone and argued that the regulating hormones must be formed by the organism itself and cannot be part of the diet.
He maintained that cholesterol could be the precursor of the actual hormone. An analogy is the fact that the vertebrate hormones adrenalin and thyroxin are metabolites of the aromatic amino acids tyrosine and phenyl-alanine, which are abundant in the diet.

It is generally believed that insects do not synthesize cholesterol or steroids. The synthesis of the steroids is blocked at the stage of squalene. However Saito et al (12) found that in the silkworm C14 acetate was incorporated into sterols as it is in mammals and that this synthesis takes place in the brain. Recently there has been experimental confirmation for the activity of cholesterol and other sterols as brain hormone (13,14). The active hormone derived from insect brains probably has similar properties to cholesterol and other sterols. Oberlander and Schneiderman (15) studied the mode of action of the brain hormone with the radioautographic technique. Under hormonal influence intense RNA synthesis was observed in the nuclei of the prothoracic glands, the target of the brain hormone. This was followed by the appearance of cytoplasmic RNA and by protein synthesis. It was assumed by the authors, that the increase in protein synthesis in the prothoracic glands was due to an increase in the synthesis of the specific enzymes necessary for the production of ecdysone.

**ECDYSONE**

This hormone is produced and released as a result of a stimulation of the prothoracic glands by the brain hormone. Fraenkel (16) in 1935, was the first to demonstrate the physiological activity of the prothoracic glands, by ligation experiments. In 1940, Fukuda
(17,18) rediscovered the glands and showed their role in the determination of the pupal moult. Williams (19) demonstrated the role of the glands in the development of the imago (adult). Starting from 500 kg. of silkworm pupae Butenandt and Karlson (20) isolated 25 mg. of crystalline hormone. It is a steroid with the empirical formula of \( C_{27}H_{44}O_6 \) (21), with one keto group in conjugation with a double bond and 5 hydroxyl groups. On first isolating the hormone, the authors identified two active fractions \( \alpha \) and \( \beta \) ecdysone, which had similar absorption spectra. Later, by counter-current distribution 5 separate fractions were isolated (22).

The idea that the insect hormone may act directly on the chromosomes was first put forward by Schneiderman and Gilbert (23). They suggested, that the nucleus could be a strategic place for hormonal action. According to biological concepts, the whole program of development is genetically fixed. The genes contain the required information governing the nature of events and processes about to take place, the timing being under hormonal control. They made the logical suggestion that the site of hormonal action should be the gene. Moreover, it was possible to test this idea since Diptera contain giant chromosomes which can be seen in cells of the salivary glands, midgut, rectum, testes and malpighian tubes. The salivary chromosomes of Drosophila and Chironomus have been favourite objects for cytological studies for many years. These chromosomes are bundles of hundreds of thousands of identical, stretched, chromosomal threads arranged side by side in register. The structure reveals a species specific linear sequence of bands, varying in width, form and structure. Swellings known as puffs occur on these chromosomes. The
bands are Feulgen positive and contain DNA. Comparative studies showed that a given position on a chromosome sometimes appeared as a puff and sometimes as a band (24). In the fruit fly many genes have been mapped on the giant salivary chromosomes. It was possible to correlate a particular gene with a particular band or puff. The gene is "active" when the position appears as a puff. This fact has very great significance as it indicates that a gene may be "active" or "inactive". It was found that only 10% of the bands are in the puffed condition. The bands of genes which were puffed varied according to the type of cell and stage of development. Certain salivary gland cells which contained chromosomes on which a particular spot was puffed, formed a granular secretion in abundance. Clever (25,26) has noted that a definite pattern of localized puffs appears at a definite time sequence, following the administration of ecdysone. It is as though the hormone, directly or indirectly, activated or depressed a few genes which in turn depressed other genes. Variations in the amount of hormone reaching the cell bring about different puff patterns. Clever's work suggested how a progressive change in differential genic activity can bring about the precise sequence of cellular differentiation characteristic of normal development. It was found that 25-30 minutes after the administration of ecdysone new puffs appeared on the giant chromosomes of the salivary glands (27). The amount of hormone required for this was very small (10^{-5} mg). Radioautography showed that only the puffs synthesized RNA. In ligated Calliphora the administration of hormone causes puparium formation. The puparium has a hardened dark brown larval cuticle. This larval
cuticle arises by a process known as sclerotization, similar to the tanning of leather by quinones. The phenol oxidase system brings about the formation of O-quinones which are incorporated into the cuticle. By using C\textsuperscript{14} labelled compounds, it has been possible to show that metabolites of tyrosine including DOPA are deposited in the cuticle. The actual sclerotizing agent was found to be N-acetyl dopamine. The steps of the process are as follows:

1) Hydroxylation of tyrosine to DOPA
2) Decarboxylation of DOPA to dopamine.
3) Acetylation of dopamine to N-acetyldopamine and polymerization (28, 29, 30).

It was found that when ecdysone was injected there was a rise in DOPA decarboxylase activity (31) and it was assumed that the action of ecdysone on the appropriate gene locus was the cause of this rise. The phenol oxidase complex is also responsible for sclerotization. Horowitz and Fling (32) showed that extracts of Drosophila were devoid of phenolase activity, but became active on standing at 0\textdegree. It was shown that the activator could be separated from phenolase, which also seemed to increase in concentration during activation. These results could be accounted for by the reaction:

Precursor + Activator \rightarrow Phenolase + 2 Activators

Administration of ecdysone to ligated larvae gave rise to the activator enzyme (33) and it was suggested, that this was a result of the formation of a new enzyme protein. It was thought, that the latter appeared as a result of the action of the hormone on certain genes.
The giant chromosomes and the puffing phenomenon are not unique for insects. The lamprich chromosomes of the amphibian oocyte undergo similar changes (34,35,36). There is also evidence that the steroid hormone, hydrocortisone, stimulates nuclear RNA synthesis during the induction of tyrosine transaminase and other transaminases in rat liver (37). Administration of estrogen to the fowl has been shown to result in production by the liver of two new proteins, phosvitin and lipo-vitellin (38). During administration of estrogen total cellular and nuclear RNA increased (39).

Though it is common belief that ecdysone activates the gene, the mode of activation is not clear. Monod et al (40) presented a model system for the control of genic action, based on the mechanism of the control of the induction of $\beta$-galactosidase in E. coli. They showed that the molecular structure of a protein is determined by a structural gene, which synthesized messenger-RNA (mRNA). This mRNA combines with the ribosomal RNA to serve as a template for protein synthesis. The production of mRNA can be initiated by acting on a certain point known as operator on the DNA strand. Several adjacent genes may depend on a single operator. This group of genes is called the operon. Besides structural genes and operator, the genome contains regulator genes in which the nucleotide sequence is similar to that in the operator gene. The regulator gene forms RNA, called suppressor-RNA, which associates reversibly with the homologous operator and blocks the synthesis of messenger-RNA. The inducer removes the suppressor, thereby liberating the operator to activate the structural
gene(s). The same mechanism has been found in the histidine operon of a mutant of *S. typhimurium* (41) in which the operator activates nine enzymes participating in histidine synthesis.

Another possibility for the mechanism of genic activation was proposed by Alfrey and Mirsky (42). They showed that most of the DNA in highly differentiated cells is inactive or "repressed" and histones play a role in repressing RNA synthesis, particularly the arginine rich histones, which combine with the DNA primer. It was shown that actinomycin D inhibits RNA synthesis that occurs on the loops of the giant lampbrush chromosomes of *Triturus viridescens*, which are known to be the site of intense RNA synthesis. This system is also inhibited by treating with arginine-rich histones. Reports have appeared lately concerning the effect of ecdysone on mammalian cells. An increase in $Q_0$ was observed by incubating ecdysone with slices of cardiac muscle (43) and an increase in leucine $\text{C}^{14}$ incorporation into the TCA insoluble fraction of a cell free protein-synthesizing system from mammalian liver (44).

**The Juvenile Hormone.**

The juvenile hormone is produced in the corpora allata and determines the larval character of the moult. When the corpora allata are active the larva moults and remains at the larval stage. At a low level of hormone a pupa emerges and in the absence of hormone an adult. Experiments involving transplantation and removal of the glands demonstrated the endocrine role of the corpora allata (45). During the last larval instar the glands are not active. Additional larval moults may be obtained by transplanting glands to
last instar larvae. As many as six additional larval moults have been obtained in *T. molitor*, the larvae reaching a very large size (46). Diminutive pupae and adults are formed when the glands are removed from early stage larvae (47).

In 1956, Williams (48) detected a rich source of the hormone in the adult male moth of *Hyalophora cecropia*. The hormone could be extracted with ether or alcohol-ether. It is interesting to note that extracts from several mammalian tissues, especially from thymus, show juvenile hormone activity. Extracts from a wide range of phyla, from both the plant and animal kingdoms, have been found to be active in this respect (49). These phyla include *Cnidaria* (class: *Hydrozoa*); *Rhynchozoa* (class *Anoplia*); *Ectopodota* (classes: *Ctenostomata*, *Cheilostomata*); *Annelida* (class: *Polychaeta*, *Oligochaeta*); *Arthropoda* (classes: *Insecta*, *Malacostraca*, *Arachnida*); *Mollusca* (classes: *Pelecypoda*, *Gastropoda*); *Echinodermata* (class: *Holothuroidea*).

Activity has also been found in extracts of yeasts, moulds and bacteria (50,51). The faeces of *T. molitor* possess hormonal activity (52). The active substance in this case was identified as farnesol (53). Its activity, however, is not as high as the activity of purified extract from cecropia. Purified preparations of the hormone are $10^6$ fold as active as farnesol (54).

Farnesol is a straight chain terpene primary alcohol, composed of three isoprene units. It has four possible steric isomers, occurring at the $\Delta^2$ and $\Delta^6$ positions of the molecule. It was shown (55) that only isomers of the transconfiguration at the $\Delta^6$ position were active. Wigglesworth (56) reported that the methyl-ether of
Farnesol and N\textsubscript{1} N-diethylfarnesylamine are much more active than farnesol. Injection of 0.1 μg per insect, of farnesyl-methyl-ether has the value of 10,000 Tenebrio units in the biological assay, in comparison to 19 μg of farnesol, which has a value of 50 and 15 μg of cecropia crude oil extract which has the value of 66 (8).

It is thought likely that the juvenile hormone is a derivative of farnesol. There is a possibility that the active substances that were extracted from many tissues are precursors of the "real" juvenile hormone. If this is the case, they must be transformed by the cells they affect and not elsewhere, since in the wax test and in the Tenebrio test (57) their juvenilizing action can be restricted to the area of application.

There is some evidence that farnesol and its derivatives copy the action of the brain hormone by activating the prothoracic glands in brainless pupae (14). Farnesol and its derivatives as well as extracts with juvenile hormone activity are known to act as gonadotropic hormones in the adult insect. The insect may use two hormones to control the prothoracic glands during larval life. The juvenile hormone causes the prothoracic glands to produce a low level of ecdysone, required for growth. Periodically the brain hormone superactivates the prothoracic glands, which respond by releasing a large amount of ecdysone. Then moulting occurs.

The fact that terpenes have hormonal activity in insects is not unique. Terpenes are known to have biological activity similar to estrogens in mammals. Their action may be potent as in the case of diethylstilbestrol.
METAMORPHOSIS AND CELL DIFFERENTIATION

Since in insects most adult tissues arise from larval cells, the study of the biochemistry of insect metamorphosis is essentially the study of the problem of cell differentiation. Cell differentiation has been followed mainly in embryonic tissues. In metamorphosis, however, the changes in form and function of cells is post-embryonic.

The differentiation of cells is most familiarly associated with cell division. In mammals, for example, life starts as a single cell, a fertilized egg. Through a series of divisions the cellular building blocks of the animal are formed. During development, the first cells to arise seem to be identical, but soon diversities appear. At first the differences may be slight but later highly specialized types of cells occur. About one hundred different kinds of normal cells can be distinguished by structure and function.

Cellular differentiation can also occur in the absence of cell division. Differentiation may occur as cells grow older. Changes in cells also develop in response to changes in environment. Cells can be altered by contact with other types of cells. Substances diffusing into cells from the milieu as well as circulating substances such as hormones, bring about transformations in cells. Differentiation without cell division is evident in the life-cycle of the slime mould Dictyostelium, which exists at one stage as a colony of separate creeping amoebae. The latter are formed from similar cells by repeated divisions. When these cells have exhausted the food supply in the medium they aggregate into a mass. Without further cell division the amoebae form an upright stalk and a capsule containing spores.
Apparently identical cells have differentiated in form as well as in function without cell division.

In the case of the Protozoan, *Paramecium* two identical asymmetric patterns of complex structure are created from one, without dedifferentiation. All the differentiated parts of the original cell are maintained. Differentiation has occurred within the cell prior to division. This could not possibly be achieved by a mere transverse cut, for that divides it into two very different halves. The cell, however, achieves production of identical daughter cells by a complicated reproduction of all its precisely localized structures with distribution in such a way as to reconstitute the original pattern in both daughter cells.

In *Tenebrio*, the cells of the dorsal and the ventral abdominal wall of the pupa are alike. However, in the adult beetle the cells of the dorsal abdominal wall form a delicate membrane, while the cells of the ventral abdominal wall form thick horny plates. The cells of the dorsal abdominal wall of the pupa differentiate to the adult tissue without cell division (58). This tissue was, therefore, chosen for some of the *in vitro* and *in vivo* experiments, to be described.

There is every reason to believe, that cells arising by ordinary division from a common ancestral cell have exactly the same set of genes. In the process of cell division, each chromosome replicates exactly, and one of the two identical daughter chromosomes passes to each daughter cell. Thus all the cells of the body, descended from the same egg cell, would have the same set of chromosomes and genes. The cell differentiation arising during development, therefore, appears not to be due to possession of different genes.
Gurdon (59) has shown that when nuclei from differentiated intestinal epithelial cells of larval *Xenopus* tadpoles are injected into enucleated eggs, the eggs develop to adult stages. It appears that the pattern of sequential gene action can be recycled in nuclei from the intestinal epithelial cells after they have undergone repeated mitotic divisions, since the daughter nuclei participate in the various cellular differentiations that occur in the embryo.

There is also biochemical evidence on the chromosomal level that the chromosomes replicate into two identical daughter chromosomes. In 1957, Taylor et al. (60) using the radioautographic technique, showed clearly, the semi-conservative manner of chromosomal DNA replication in root cells of *Vicia* labelled with thymine $H^3$. This has been confirmed with many other plant species. It has also been shown in mammals and insects (61). The first evidence that replication might be semi-conservative at the molecular level was provided by Levinthal (1956) (62). In an autoradiographic study of the distribution of $B^{32}$-labelled DNA from phage $T_2$, he obtained evidence that about 40% of the DNA, which remained in one piece when the phage was broken by osmotic shock, was replicated semi-conservatively. Meselson et al. (1958) (63) used *E.coli* grown for a number of generations in $N^{15}$-labelled medium. The heavy DNA of the cells could be separated from the $N^{14}$-containing DNA in a cesium chloride gradient at high centrifugal force. With this technique they demonstrated the semi-conservative manner of DNA replication. Similar results were obtained in mammals (64, 65) phages (86) and algae (67). The explanation for DNA replication on the molecular level is based
on the Watson-Crick model for DNA (68). This model represents a simple double helical array, consisting of two polynucleotide strands of opposite polarity. The strands are held in helical configuration by the H-bonds formed between complementary pairs: A-T and G-C. The replication is facilitated by separating the strands and allowing the complementary strands to polymerise on each of the pre-existing chains. This results in the formation of two DNA molecules identical with the parent molecule (69).

Cell differences can be correlated with the activity of different genes, as if genes existed in only two alternative states active and inactive. The same gene can be active to varying degrees and such differences in genic activity are also important in cellular differentiation. This has been proven by Markert (70) and others, with the enzyme lactate dehydrogenase. This enzyme exists in a group of somewhat different forms, constituting what is called a set of isozymes. Lactate dehydrogenase occurs as five isozymes, all of which usually occur in the same cell. Cells of different tissues or organs, or the same tissue, or organ at different stages of development have different proportions of the five isozymes. This variation in the proportions of the isozymes is due to differences in the relative activities of two genes. These genes are responsible for the production of two polypeptides, which constitute the building blocks of the enzyme. Each enzyme molecule is a tetramer, consisting of four polypeptides. The four may all be one kind of polypeptide, or all the other kind, or any of the three possibilities of combining the two kinds of polypeptides in groups of four as follows: 3:1, 2:2 or 1:3. The
relative amounts of the five isozymes formed when the two kinds of polypeptides are mixed in varying proportions in vitro, is exactly what would be expected by chance combinations. As the polypeptides are genic products, the different proportions of the isozymes found in different kinds of cells appear to be due to relative differences of the two genes involved.

The characteristic of differentiation is the end product, the protein. A reticulocyte will synthesize haemoglobin, liver cells produce glucose-6-phosphatase and albumin, kidney cells differentiate to make L-amino acid oxidase and muscle cells, myosin. This does not mean, however, that similar genes are not active in different cells, since one finds many enzymes which are common to many types of cells. Specific enzymes and structural proteins are the end product of active genes. This activity in biochemical terms, means production of a specific mRNA's on the DNA template, which serves as a template for the synthesis of specific proteins.

Since DNA and especially RNA, direct protein synthesis and their activity occurs prior to protein synthesis, it appeared profitable to examine the role of nucleic acids during metamorphosis. In the present work a study on nucleic acid metabolism during metamorphosis, when larval tissues differentiate into adult tissues of a beetle, was carried out on T. molitor.
BIOCHEMISTRY OF INSECT METAMORPHOSIS

The biochemistry of insect metamorphosis has been studied from three different aspects. The first one is descriptive - the study of fluctuations of metabolites and activity of enzymes in the course of metamorphosis. The second is physiological - involving the hormones which govern the whole process and the details of hormonal control. The third is a combination of the first two. Some results are difficult to evaluate, since the insect studied most extensively has been the moth H. cecropia which hibernates during metamorphosis. It is, therefore, difficult to distinguish whether certain changes are due to hibernation, or to metamorphosis.

Respiratory Enzymes and Respiration.

Krogh (1914) (71) discovered the characteristic U-shaped curve for oxygen consumption during metamorphosis. The insect studied was the mealworm, Tenebrio molitor. This type of curve has been found in other holometabolous insects such as D. melanogaster (72-74), and in many other species of insects (75-81). Taylor (1927) (76) noted, that the depth of the respiratory curve was influenced by sex and the duration of the pupal stage. Insects which have a short pupal stage have a deep U-shaped curve, while those with a longer pupal stage have a shallow U-shaped curve. He also observed that females use oxygen at a greater rate than do males of the same age.

Numerous explanations have been advanced to account for the U-shaped respiratory curve. Fink (1925) (82) correlated the U-shaped oxygen consumption curve with the process of histolysis of larval
tissues and histogenesis of adult tissues during metamorphosis. The descending part of the curve indicates histolysis, the ascending part histogenesis and the lowest part, the least amount of tissue organization. However, Buck (1953) (83) stated, that there is considerable evidence showing, that even though the intensity of morphogenetic change varies within the pupal period, histolysis and histogenesis proceed concurrently, without either being markedly predominant at any particular stage of pupation.

Many workers correlated the U-shaped respiratory curve with similar changes in enzyme activity. Wolsky (1938) found a U-shaped activity curve for cytochrome oxidase during the metamorphosis of *D. melanogaster* and related it to oxygen consumption (74). The same correlation was found by Williams (1950) with the moth *H. cecropia* (84), but one has to bear in mind that the *cecropia* silkworm undergoes diapause during pupation. Sacktor (1951) (85) with the house-fly and Ludwig (1953) (86) with the Japanese beetle, *P. japonica*, found U-shaped curves for both cytochrome oxidase activity and respiratory metabolism. These experiments indicate that cytochrome oxidase may be a rate limiting enzyme. Ito (1954) studied the U-shaped curve of respiration in the silkworm *B. mori* (80). After correlating this activity to the activity of cytochrome oxidase and succinic dehydrogenase, he concluded that these curves could not be correlated with respiratory metabolism. (87). A most extensive study of the cytochrome system in *H. cecropia* during diapause and development, was carried out in Williams Laboratory (88-100). A rise in oxygen consumption a week prior to the
termination of diapause was observed. It was first suggested, that
this increase in metabolism is alone sufficient to explain the
onset of imaginal differentiation. This could not be the case,
however, since injury also causes an increase in respiratory met­
obolism to levels characteristic of post-diapausal insects, but
does not initiate adult development.

The quantitative variations of succino-oxidase cytochromes
b, c, (a + a₃) and b₅ were studied in H. cecropia. Throughout metamor­
phosis all tissues, with the exception of the pneumatic muscles,
show great variations in cytochrome concentrations. During diapause
cytochromes b and c are not detectable, but in the larval and adult
stages they are present in moderate to abundant concentration.
Cytochromes b and c reappear with the initiation of imaginal
differentiation (end of diapause) (90-95). It was also observed
that during active metamorphosis the insects are sensitive to cyanide
and carbon monoxide, (96) which inhibit cytochrome oxidase. Diapausing
pupae are not sensitive. Microsomal cytochrome b₅ is thought to be
the terminal oxidase (97). Chefurka and Williams (98) noted changes
in the prosthetic groups of flavoproteins, with maximal concentration
during diapause and decrease during adult development. Wolsky (101)
in 1941, observed a correlation between the activity of succinic dehy­
drogenase and oxygen consumption in D. melanogaster. Succinic dehy­
drogenase was U-shaped during metamorphosis. Similar results were
found by Ludwig and Brasa with Tenebrio, the Japanese beetle and the
house-fly (102-104). They concluded, that since its activity is
very low, it could be a limiting factor for respiration.
Agrell (1949) (105) studied the activity of various dehydrogenase enzymes during metamorphosis of the blow fly, Calliphora erythrocephala. He reported that malic, succinic, citric and glutamic dehydrogenases all showed typical U-shaped curves. He concluded, that the form of respiratory curve is due to a change from predominating histolysis, especially of the larval fat body, into predominating histogenesis of the imaginal musculature.

In T. molitor (104,106) U-shaped curves were shown for the activity of malic and succinic dehydrogenases and the malic enzyme during metamorphosis. The authors pointed out, that not all the enzymes they studied exhibit this type of curve. The activity of alcohol, glucose-6-phosphate, glutamic, lactic and \( \alpha \)-glycerophosphate dehydrogenases did not follow a U-shaped curve. On the other hand, in the house fly, \( \alpha \)-glycerophosphate and alcohol dehydrogenases showed a U-shaped curve during metamorphosis (104). The activity of malic, succinic and isocitric dehydrogenases, the malic enzyme, and cytochrome oxidase, also showed a U-shaped curve during metamorphosis of the house-fly.

**Nitrogen, Amino-acid and Protein Metabolism**

The pupa is a closed system except for the exchange of gases. Since only traces of ammonia are produced in the pupa (107), the total amount of nitrogen should be relatively constant. The amino acids needed in histogenesis have to be provided either by the pool of free amino acids, or by breakdown of protein reserves. The latter occurs to a great extent. Even the proteins of the cuticle are digested before the moult by the moulting fluid, which is rich in proteases,
and the amino acids are reabsorbed (108).

The numerous investigations on the fluctuations in nitrogen and in amino acid content have not revealed points of special significance. The results are more or less those that are to be expected on the basis of histolysis, histogenesis and protein biosynthesis. The total amount of nitrogen during pupation was studied in Tenebrio (109) and other insects (110-112) and was found to be constant. In the haemolymph of Bombyx there is a decrease of 35-50% in total nitrogen concentration during pupal life (113-115). However, in Deilephila pupae, which go through the extended diapause, the over-all drop in nitrogen is about 75%. The most striking feature of nitrogen metabolism in metamorphosis is the relative constancy of both non-protein nitrogen and amino nitrogen in pupal blood (113-117). The concentration of uric acid in blood in general is close to the saturation value in water and shows no marked change during pupal life (118-120).

One striking feature of insect haemolymph is the high content of free amino acids, first recognized by Florkin in 1937 (121) and regarded to be a taxonomic character for the insect group. There are considerable differences in the actual values for different species; the values range from 3 to 24 gm per litre haemolymph, as compared with 0.02 to 0.9 gm. per litre of plasma for vertebrates (122). In metamorphosis great variations in the quantitative sometimes also in the qualitative, content of amino acids are observed. Wyatt et al (123) noticed in the silkworm, that at the time of pupal moult there is a temporary increase of alanine and glycine, which then progressively falls again during pupal life. Glutamic acid increases
during the pupal moult and stays at a high level in the pupa. Proline and lysine are found at higher concentration in pupal than in larval life. The adult blood shows a fall in lysine, glycine and histidine concentrations. Amanieux et al (124) found an increase in the methionine content in pupae as compared to larvae. Patterson (1957) (125) measured the pool of free amino nitrogen during the metamorphosis of T. molitor. The level of total free amino nitrogen in pupal tissue varied little during metamorphosis in spite of the tremendous morphological changes, which the insects undergo at this period of their existence. The average total free amino acid in Tenebrio varies between 250-300 µg/pupa (pupae weigh 110-120 mg). Two thirds of the total amino acid is in the blood. Further examination of the variation in the titre of sixteen free amino acids during pupation of Tenebrio showed no significant changes. A small increase in total nitrogen and in individual amino acids, observed at the beginning and the end of pupation, was thought to be due to absorption of pupal cuticle protein during ecdysis. The following amino-acids showed a downward trend: Aspartic acid, glycine, glutamic acid, proline, glutamine, erginine, lysine, valine, methionine and tryptophan. Tyrosine gradually reached a high concentration. The concentration of alanine and leucine remained unchanged. Serine and threonine slightly increased in concentration. Towards the end of metamorphosis, the concentration of these two amino acids slightly decreased.

The α-amino nitrogen content of pupal tissue is approximately constant during metamorphosis of other insects (121, 116, 126).
Agrell in 1949 (127), made a semi-quantitative investigation of the free amino-acids in Calliphora pupal breis and also observed the approximately constant size of the free amino-acid pool. He considered the concentration of free amino acids to be maintained by a balance of protein synthesis and protein breakdown. According to him, quantities in excess of an equilibrium titre of free amino-acids, were oxidized especially at the beginning and end of metamorphosis. These periods of more intensive amino-acid oxidation corresponded with the peaks of the U-shaped oxygen utilization and various amino acid dehydrogenase activity curves. A serious objection to this hypothesis is, that no more than a small fraction of the total respiration could be accounted for by oxidation of the amino acids in the haemolymph by dehydrogenases. As mentioned before, lysis and protein synthesis proceed concurrently, without either being markedly predominant at any particular stage.

The levels of amino acids during cocoon spinning have been studied (120, 128-130). Florkin found a decrease during spinning, followed by a return to the former high level in pupae. Fukuda compared the amino-acidaemia of glandectomized silkworms with that of normal development. The amino acid concentration in glandectomized animals is slightly higher during larval life and was restored at the time of pupal moult. In the period between the last defaecation and the pupal moult, the concentration of glycine falls in normal animals. In glandectomized animals, there is an increase in concentration of glycine in comparison to the control. During spinning, serine concentration also falls, but during the pupal life, the amount of serine
increases. In *Ephestia* (131), the amount of ninhydrin positive substances increases just after pupation and then progressively declines until the emergence of the adult. Ornithine, β-alanine and phenylalanine are found only in the pupal stage. The concentration of tryptophan in *Ephestia* was studied in the (a+) and (a) strains in connection with the biosynthesis of ommochromes and was found to reach its maximum peak in the pupa.

Studies on the amino acid composition of the haemolymph of the blow fly *Calliphora* were carried out by Hackman (132). A quantitative difference was found only for hydroxyproline, which is present in larval and prepupal blood and is absent in the pupa. The total amount of amino acid falls from 6.6 mg/ml of larval blood to 3.3 mg/ml in the prepupae, then rises again to 4.6 mg/ml in early pupal blood. The greatest decrease occurs in the concentration of alanine, glutamic acid, glutamine, glycine, proline and especially tyrosine. These changes reflect puparium formation, during which, biosynthetic processes are responsible for the formation of new cuticle and sclerotization. Glutamic acid may be used for the formation of glucosamine, the precursor of chitin. The cuticle proteins are rich in proline and tyrosine.

Changes in blood proteins during larval life and metamorphosis have been reported for *H. cecropia* (133). By immunological methods, nine different proteins can be distinguished. Three are present in constant concentration through larval and pupal life; three others appear in the last larval instar. The physiological significance of these proteins is not clear.
On the basis of the observation that the concentration of haemolymph peptides increases during the pupal stages in Calliphora, Agreij (134) considered whether peptides could be incorporated as units into proteins. Present knowledge excludes this possibility. The rise in peptides may occur as a result of the incomplete breakdown of protein.

Telfer (135) by immunological means showed that one protein exists only in female blood in high concentration. It is taken by the yolk during egg formation.

Skinner (136) studied the incorporation of C\textsuperscript{14} valine into protein of cecropia pupae. All the tissues of the diapausing pupae incorporated amino acids into their proteins, though the labelling of the blood proteins was slight. Within the first few days after initiation of adult development, incorporation rates of valine are increased three to five times into epithelium, midgut, fat body and several hundred times into blood. In epithelium the rate of incorporation remains constant in the late stages of pupal development. In contrast, several days before adult emergence the rate of labelling of blood proteins increases further, while the rate of fat body proteins decreases sharply. The rate of synthesis of midgut protein is high even during diapause.

At a later stage of development when the rate of protein synthesis in all tissues is much faster, there is an increase in radioactivity in the blood proteins. Such incorporation is always preceded by a lag period of several hours. It was assumed that this lag is due to the fact that proteins are synthesized within the cells
from which they are then secreted.

Injection of ecdysone to diapausing animals initiates adult development and increases amino acid incorporation to the level characteristic of the developing adult. Injury, however, increased amino acid incorporation in all tissues studied without adult development. This increase is indistinguishable from the early stage of development.

Recently, Sekeris and Karlson (1964) (137) followed the formation of DOPA-decarboxylase in the prepupae of Calliphora erythrocephala. This enzyme is absent in the larvae and is induced by the action of ecdysone, which increases in its titre during this period. These authors demonstrated that pupation of blowfly larvae can be delayed for a considerable time by the injection of streptomycin, chloromycetin, puromycin, mitomycin, actinomycin and fluoro-deoxy-cytidine. The induction of the enzyme, DOPA-decarboxylase, is inhibited by these metabolic inhibitors. All the above mentioned inhibitors are known to act on the chain between DNA (gene) and protein. The authors argued that inhibitors of nucleic acid and protein biosynthesis will inhibit hormone action. Ecdysone is responsible for puparium formation in Calliphora. The puparium is delayed especially if the inhibitors are administered 25 hours prior to puparium formation. Some hours later, a sharp increase in hormone concentration is seen. This continues up to the time when the new pupa is formed. These results were explained by the fact that a specific protein or messenger-RNA or both, which are produced as a result of induction by ecdysone, are inhibited.
An objection to this interpretation is the fact that prior to ecdysis there is very active epidermal cell division for which ecdysone is responsible. This hormone causes enlargement of the epidermal cells followed by mitosis and the secretion of a new cuticle. In the special case of puparium formation in Diptera, it is also responsible for the tanning of larval cuticle after cell division (138). The specific enzymes and structural proteins of the puparium are the product of the epidermal daughter cells of the last larval instar. Therefore, it is not likely that the metabolic inhibitors, mentioned above, act at the stage between the specific gene (DNA) and the specific protein, in this case the DOPA-decarboxylase. They may act in a non-specific manner as inhibitors of RNA and protein synthesis, which will stop or delay epidermal cell division. This would result in indirect inhibition of production of DOPA-decarboxylase and puparium formation. According to Sekeris and Karlson, actinomycin-D is the most potent inhibitor of puparium formation. The concentration of drug they used was extremely high, 0.02γ/larva. The average weight of the larvae is only a few milligrams. It was shown that actinomycin-D in high concentrations inhibits cell division, in a manner similar to colchicine, by arresting metaphase and producing C-mitosis (139). The net results obtained by Sekeris and Karlson may therefore be due to a secondary effect, namely, the arrest of mitosis and delay in production of daughter cells, which are responsible for puparium formation.

The same argument is valid for the action of mitomycin and other inhibitors. It has been shown (140,141) that at certain concentrations of mitomycin, several strains of bacteria enlarge but do not
divide. Puromycin inhibits the amino acid activating enzymes and blocks the formation of aminoacyl-transfer-RNA. It is an inhibitor of bacterial and animal cell division (142).

In some Lepidopteran insects the prepupae spin silk cocoons. The silk is secreted from the silk glands. The mechanism of silk formation, biosynthesis of silk, the incorporation of radio-isotopes into silk, the analysis of the molecular weight and the amino acid composition and sequence in silk have been studied. Bombyx mori has been used extensively as it is of great economic importance. (143).

CARBOHYDRATE AND FAT METABOLISM

The use of stored material for energy needs during metamorphosis is a consequence of the fact that no food is ingested during that period. Fat and carbohydrate reserves accumulate in varying extents in different species.

The interrelationship between carbohydrate and fat in metamorphosis is complex and differs in various insects. There is an increase in total reducing value at the pupal moult. (144, 145). The total concentration of reducing substances, in the blood and in the whole body, generally reaches a peak between the time feeding ceases and the pupal period commences. There is a decrease in reducing value throughout the pupal stage. The level of fermentable sugar follows a similar course. (138, 146). A secondary peak of reducing substances has been observed close to the time of adult emergence, in several insects (147-149).

The major carbohydrate constituent of insect blood has been identified as the disaccharide trehalose. (150-152). It has been
found in all insects examined. Changes in trehalose concentration during metamorphosis have been followed in many insect species. Duchâteau et al (153) observed, in Bombyx, that the concentration falls from 250-600 mg per milliliter in the fifth larval instar, (last instar) to almost nil at pupation. The concentration then rises again during adult development. The same pattern of changes was observed by Wyatt and Kalf (154) in cecropia silkworm, but not in Deilephila euphorbiae (155).

In many insects when the last larval instar ceases to eat, before metamorphosis, there is an increase in glycogen which corresponds to the decrease in the trehalose content during this period (154, 156). It has been suggested that insects may carry out net conversion of fat into carbohydrate (157-159). Since vertebrate animals are not known at present to possess any pathway for the net conversion of fatty acids into carbohydrates, the existence of such a pathway in a major invertebrate group would be of great interest. In micro-organisms and higher plants the utilization of fats and metabolically related substances such as acetate, for carbohydrate synthesis is well established (160). Passey and Fairbairn (161) have reported the occurrence of such a conversion in the nematode Ascaris, though the mechanism has not been elucidated.

The earliest assertion for the conversion of fat into carbohydrate in an insect was based on the observation of Courveur, in 1895, (162). He found that after the cessation of food intake by Bombyx, glycogen increased concomitantly with decrease in lipids. This finding has been verified by experiments with Bombyx and other insects. In 1962
Bade and Wyatt (163), however, presented results of quantitative analyses of the fat and glycogen content of *cecropia* silkworms from the mature larva to the early pupa. By using carbon C\(^{14}\) they were able to show that sources other than fat can account for glycogen that is formed during pupation. According to these authors the amount of lipids decreases steadily during the prepupal period. The amounts of glycogen and free sugar decrease during spinning, then show an apparent increase about the time of pupal moult. The amount of chitin as well as the total weight of the cuticle decrease sharply just before the moult. Therefore, they assume that there is ... synthesis of glycogen and sugars by conversion of substances from the cuticle without invoking conversion of lipids. When larvae were injected with glucose-l-C\(^{14}\), just before their fourth moult, by the end of the fifth instar, the retained label was almost exclusively in the cuticle. Then, during the pupal moult, isotopic carbon was transferred to glycogen, sugars, and lipids. The newly synthesized pupal cuticle had a high specific activity similar to that of the larval cuticle. It was suggested, therefore, that a re-use of materials had taken place. Zaluska in 1959, (164) was the first to suggest, though without experimental evidence, that chitin may serve as a source of carbon for glycogen synthesis. The possibility of direct utilization of material from the larval cuticle for synthesis of pupal cuticle had already been considered by Lafon, in 1943, for *Tenebrio molitor* (165) and by Zaluska (164) for *Bombyx*.

Bade (166) examined the glyoxylate cycle in *cecropia* silkworm.
This enzymatic pathway is utilized for carbohydrate (malate) synthesis from acetate units and glyoxylate acid in micro-organisms and plants (167-169). By using acetate-2-C\textsuperscript{14} and comparing the incorporation of radio-activity in the presence of various substrates and of malonate, into the component acids of the citric acid cycle, Bade reported the following facts:

a) A decrease of radioactivity in malate when glyoxylate is added, also unlike oxaloacetate, glyoxylate is not an efficient remover of radioactive acetate. This observation suggests that malate synthetase is not active.

b) Very little C\textsuperscript{14} is incorporated into malate in the presence of malonate, suggesting that the major route of malate synthesis is via the citric acid cycle.

These results are consistent with the operation of the citric acid cycle as a major pathway of acetate metabolism in the last instar prepupae and pupae of the \textit{cecropia} silkworm.

There is a marked decrease in tissue glycogen content during metamorphosis. The decrease in Bombyx at the end of the pupal stage reaches one fourth to one fifth of the initial value. It is believed that glycogen is used by the pupa as a source of energy. Ito and Horie (171) examined the glycogen content of normal \textit{Bombyx mori} pupae, as well as in insects ligated at the mesothorax within 3 hours after pupation. The glycogen content remains at a constant level in ligated animals, but it is decreased in the normal pupae. This finding indicates that in insects as in mammals glycogen breakdown is under hormonal control.
Insects possess a high lipid content, the latter constituting 20 - 30% of their dry weight. Agrell (134) found that about 30% of the energy metabolism is covered by fat utilization, carbohydrates being metabolized only to a small extent.

A number of investigators have found values of 0.1 to 0.7 for the respiratory quotient during pupal life (77, 82, 174-176). Values of 0.1-0.3 have been reported for R.Q during metamorphosis. However, it was shown later, that in several insect species the respiratory CO₂ is given off discontinuously, in discrete bursts. Only measurements over long periods (8 hours and more) give reliable results for R.Q of 0.8-0.9 (177-179).

**PHOSPHORUS AND NUCLEIC ACID METABOLISM**

Since the pupa is a closed system the total phosphorus content of the insect does not alter during the pupal stage. This has been found to be the case in *Tenebrio* (180) and other species (181, 182). A significant difference in the amount of total phosphorus between male and female pupae has been found in *Tribolium confusum*, a species related to *Tenebrio* (183). The concentration of the TCA soluble phosphorus fraction remains more or less constant during the pupation period of *T. confusum*. In many insects inorganic phosphorus decreases in the prepupae, remains constant during half the pupal period or increases only slightly. It then decreases slightly at the end of the pupal stage (184, 185). Wenig, in 1959 (186), however, reported a constancy of inorganic phosphorus during the pupal stage of *Tenebrio molitor*.

Phospholipids are present in very small amounts during the
pupal stage of *T. confusum* (183) and the amount remains constant. No major change was found during the metamorphosis of *Calliphora* (182, 184). In *Bombyx* (187) a considerable change in the character of the phospholipids, with only minor variations in the amount, was observed during metamorphosis. A gradual decrease in phospholipids was observed during the pupal period of *Musca domestica* (188) and a slow rate of decrease in *Popillia japonica* (189).

The concentration of phosphoprotein phosphorus appears to be very low as compared to the other phosphorus fractions. There is no indication of variation of this fraction during the growth and metamorphosis of the tenebroid *T. confusum* (183). Similar results were obtained by measuring phosphoprotein phosphate during the embryonic stage of *Bombyx* (190).

Nucleic acid phosphorus in *Calliphora* shows no major changes during metamorphosis (182,184). A U-shaped curve for nucleic acid phosphorus was found in *Celerio euphorbiae* (191), which was more pronounced in the winter generation. In *T. confusum* nucleic acid phosphorus remains essentially constant during pupation (183).

Wyatt (192) studied the RNA/DNA ratio in the pupal wing epithelium of the *cecropia* silkworm by the incorporation of P$^{32}$ into RNA. He observed a rise in this ratio at the early stage of adult development, as well as a several fold increase in the rate of incorporation of phosphate into RNA. The proportion of P$^{32}$ in the four nucleotides is strongly suggestive of mRNA production (196). It was therefore suggested, that RNA synthesis is involved in the early processes of morphogenesis and may precede DNA synthesis and cell division. Studies
on the RNA/DNA ratio during the early stage of adult development of *H. cecropia* pupae. We show that in the early stage the ratio is increased. This finding can be attributed to increased RNA synthesis. The ratio then falls as development proceeds, presumably due to increased DNA synthesis. Incorporation of P\textsuperscript{32} into RNA rises sharply early in adult development and later declines again. Many authors have reported a decrease of total nucleic acids during early pupal life and an increase with progressive development (184, 190, 194-196).

Devi et al (197) measured the nucleic acids and free nucleotides concentration during the growth and development of the tenebroid insect *T. confusum*. They found no changes in the concentration of DNA, RNA free ribonucleotides and free deoxycytidyluctotides during metamorphosis of this insect.

Linzen and Wyatt (198) measured the nucleic acid content of tissues of the *cecropia* silkworm pupae. The level of total nucleic acids, giving RNA by difference, have been determined in the wing tissue and fat body in diapause and at an early stage of imaginal development. It was found that in the fat body, the ratio of tissue-residue weight to DNA varies in proportion to the fresh weight of the individual pupae. This finding was interpreted on the basis of a correlation of cell size with animal size. The wing tissue does not show such a relationship. In the wing tissue, the level of DNA approximately doubles, and that of RNA rises some four fold, during the transition from diapause to the second day of adult development. In the fat body, there is no significant change in the level of either type of nucleic acid.
Patterson (125) measured DNA and RNA content during the metamorphosis of *T. molitor*. The DNA content appeared to be evenly distributed about a mean value of 140 μg/pupa. The average values for RNA fell from about 510 μg/pupa, at the beginning of pupal life, to about 310 μg/pupa half way through the pupal life and rose to about 570 μg in the end of the pupal period. The ratio RNA/DNA also exhibited a U-shaped variation during metamorphosis. Patterson assumed on the basis of the above observation that the number of cells per animal is not changed during metamorphosis since there is a constant concentration of DNA. On the other hand, the high level of RNA at the beginning and at the end of metamorphosis was explained as being due to a high level of RNA per cell. Therefore, Patterson suggested that these two periods of development might well be associated with intensive protein synthesis.

**BIOSYNTHESIS OF PIGMENTS**

In certain Lepidopteran insects, phenoxazine pigments known as ommochromes, are synthesized and deposited in the cuticle of the caterpillar shortly before pupation. These pigments are also deposited during pupation in the ommatidia of the compound eyes in *Diptera* and *Lepidoptera*. This phenomenon was correlated with changes in behaviour and is of selectional value for the species. It was shown, that this process is under the control of ecdysone, by ligation experiments as well as by direct injection of ecdysone (199).

Ommochromes are metabolites of tryptophan. The elucidation of the pathway of synthesis of these pigments is the result of investigation not only of biochemists, but also of geneticists,
including Kühn who studied the eye-colour mutants of *Ephestia*, and Tatum, Beadle and Ephrussi, whose names are associated with the biochemical approach to genetic analysis.

Strains occurred in *Drosophila* and *Ephestia* differing from the wild type by the mutation of a single gene \( v^+ v \), in which the normal black or brown coloration of the eyes was replaced by red or yellow. Implantation of organs or injection of extracts of the wild type insects would correct the pigment deficiency. The active substrate of the wild type was identified as kynurenine, a metabolite of tryptophan. In the \( v \) mutant tryptophan accumulates (200-202). A second mutant \( (cn) \) occurs in *Drosophila*, in which pigment synthesis is restored by the injection of 3-hydroxy-kynurenine but not kynurenine. The gene which controls its formation acts at a stage later than the \( v^+ \) gene. The pathway of ommochrome synthesis can thus be written as follows:

\[
\text{Tryptophan} \xrightarrow{(v^+\text{gene})} \text{Kynurenine} \xrightarrow{(cn^+\text{gene})} 3\text{-Hydroxykynurenine}
\]

A mutant, "white - 1", of *Bombyx* has also been found to lack the enzyme which converts kynurenine to 3-hydroxykynurenine, so that kynurenine accumulates, reaching a concentration of 0.3-0.5 mg/g fresh weight in the pupa (201).

Further confirmation of the above observations has been provided by the isolation of isotopically labelled pigments following the injection of either \( C^{14} \)-tryptophan or \( C^{14} \)-kynurenine (200,203).

The oxidation of tryptophan to kynurenine is believed to take place in two steps, the first of which is the formation of formyl-kynurenine, which is catalyzed by peroxidase. Formic acid
is then split from formylkynurenine by the enzyme kynurenine formidase, which has been demonstrated in cell-free extracts of Drosophila (204). The latter enzyme appeared to be equally active in mutants and the wild type. Therefore, it may be concluded that it is the production of peroxidase which is controlled by the v+ gene.

Some of the ommochrome pigments have been isolated and their structure has been identified (204). It has been shown that xanthommatin and a DOPA-melanin are formed by the action of Calliphora phenolase on a mixture of 3-hydroxy kynurenine and DOPA 3-hydroxykynurenine is not itself a substrate for phenolase. Its oxidation to the phenoazaine dyestuff xanthommatin is apparently the result of the action of DOPA-quinone formed as an intermediate in the phenolase reaction.

Inagami (205) observed the formation of "red melanin" in mutants of the silkworm. The body fluid of the mutant rb turns red on the exposure to air, instead of black as in the normal insect. This unusual pigment formation has been related to the abnormal accumulation of 3-hydroxykynurenine in the mutant (205). The latter compound disappears during pigment formation and it seems likely that the synthesis of yellow or red ommochromes in blood is responsible for the unusual colouration in the mutant. The final colour may be achieved by a model system comprising potato phenolase acting on a mixture of 3-hydroxykynurenine and DOPA, varied from yellow, through red and brown, to black, depending on the relative proportions of the two substrates added. (206).
MATERIALS AND METHODS

MAINTENANCE OF ANIMALS

_T. molitor_ larvae were maintained at room temperature on a diet of wheat-bran and oat-flakes (2:1) (125). Pieces of carrot were added once a week to provide moisture. Prepupae were collected from the culture and kept in separate containers. The pupae which emerged daily between 5 p.m. and 10 a.m., were placed in dated specimen beakers and were considered as first day pupae. These were incubated in a highly humid atmosphere at 28°C. Under these conditions pupation lasted seven days, the adults emerging on the eighth day. Adults were removed within 17 hours of emergence and transferred to dated beakers containing the diet and water-soaked cotton wool.

Rats and tumour-bearing animals had free access to Purina chow and water up to the time of killing.

CHEMICALS

All common chemicals were of "Reagent Grade" quality and were used without further purification. Adenine-8-C\(^{14}\) sulphate hemihydrate, glycine-1-C\(^{14}\), glucose-UC\(^{14}\) and thymidine-2-C\(^{14}\) were purchased from the Radiochemical Centre, Amersham, England. Uridine-2-C\(^{14}\) was obtained from New England Nuclear Corporation, Boston, Massachusetts, U.S.A. Sodium-formate-C\(^{14}\) was purchased from Picker Nuclear, White Plains, N.Y., U.S.A. The sarkomycin was a gift from Banyu Pharmaceutical Co., Ltd. Tokyo, Japan. Colchicine was purchased from the Abbott Laboratories, Montreal, Canada. Actinomycin-D was a gift from the Merck Laboratories, Montreal, Canada. Mescaline hydrochloride was a product
of the Nutritional Biochemical Corporation. Farnesol was purchased from L. Light and Co. Ltd., Colnbrook, Bucks., England. Calf thymus DNA was a gift from Dr. Sung. It was prepared according to Kay, Simmons and Dounce. (207)

PREPARATIONS OF SOLUTIONS

All solutions with the exception of colchicine and actinomycin were prepared at appropriate concentrations in distilled water. Solutions of glucose and radioactive compounds were stored at -20°C. Colchicine and actinomycin were dissolved in water for in vitro experiments and in normal saline for in vivo experiments. Fresh solutions of colchicine and sarkomycin were prepared for each experiment. Actinomycin-D was dissolved in normal saline.

PREPARATIONS OF TISSUES

Pupae weighing 110-120 mg were used. For in vitro experiments the head and thorax were removed. The dorsal abdominal slice was obtained by means of a Stadie-Riggs tissue slicer. Three such slices were used per incubation flask. Pupae were injected by means of Hamilton micro-syringes.

Ehrlich ascites carcinoma cells were grown and prepared as described by Johnstone and Scholefield (208). Packed cells were suspended in isotonic Krebs-Ringer solution (209) to yield 1:10 dilution. One ml of such a suspension was used per three ml. of incubation medium.

Rat spleen slices and regenerating rat liver slices were prepared from male Wistar albino rats weighing about 250 grammes. Partial hepatectomy was performed under ether anaesthesia according
to Higgins and Anderson (210). Twenty-four hours after the operation the animals were killed by a blow on the head and the liver was excised, chilled rapidly on crushed ice and sliced.

**INCUBATION METHODS**

Unless otherwise stated, all incubations were carried out at 37°C in Warburg flasks to ensure a controlled gas phase. The flasks were gassed for ten minutes. Radioactive compounds were tipped into the main compartment of the flask from the side arm, after ten minutes of thermal equilibration. Measurements of glycolysis and respiration were carried out by the manometric technique (209) and the results expressed as microlitres of oxygen or carbon-dioxide evolved per mg. dry weight of tissue. All incubations were stopped by rapidly cooling the flasks in crushed ice.

Most incubations were carried out in Krebs-Ringer solution 145 mM NaCl; 5.8 mM KCl; 1.5 mM MgSO₄ and 3.6 mM CaCl₂, in a final volume of 1 ml. When phosphate buffer pH 7.4 was used 10 mM phosphate was added to the final volume to avoid precipitation of calcium-phosphate. In experiments where the buffer was bicarbonate pH 7.4, the latter was added at a level of 25 mM sodium bicarbonate. This was saturated with either O₂/CO₂ (95:5), or commercially purified N₂/CO₂ (95:5). For incubating Ehrlich ascites cells calcium-free Krebs-Ringer was used in a final volume of 3 ml. 5.8 mM KH₂PO₄ was added to the Ringer solution when these cells were incubated with bicarbonate buffer.

**RADIOACTIVITY MEASUREMENTS**

All samples were counted with a thin window gas flow...
counter (20% efficiency) attached to a Tracerlab scalar. Samples were corrected for background. Corrections for self absorption were applied only for C\textsubscript{14}O\textsubscript{2} measurements. Protein samples weighed about 10 mg or less. A self absorption curve for insect protein showed that at this weight the self absorption is negligible. Self absorption corrections were not applied to other samples as these were infinitely thin.

**RADIOASSAY OF RESPIRATORY CARBON DIOXIDE**

The incubation was stopped by placing the flasks in crushed ice. The filter papers from the centre wells were then transferred to graduated centrifuge tubes containing 0.2 ml 1.3 per cent sodium carbonate solution added as carrier. The centre wells were washed four times with distilled water, the washings transferred to the centrifuge tubes, and the volume was made up to 6. ml. After stoppering the tubes tightly, they were left overnight to ensure elution of the K\textsubscript{2}C\textsubscript{14}O\textsubscript{3} from the filter papers. The filter papers were removed and washed with distilled water, the latter being collected into the tubes, which had contained the filter papers. 0.2 ml 2M ammonium chloride and 0.5 ml. 20 per cent barium chloride were added to precipitate the carbonate as barium carbonate. The precipitate was washed twice with distilled water, once with acetone and finally resuspended in 0.4 ml. of acetone. The suspension was then quantitatively transferred to weighed aluminium planchets, dried and counted. Corrections were applied for self absorption.

**DETERMINATION OF RADIOACTIVITY IN THE ETHANOL SOLUBLE FRACTION, PROTEINS AND TOTAL NUCLEIC ACIDS**

For the \textit{in vitro} estimation of the radioactivity incorporated
into the ethanol soluble fraction and protein, a modification of the method first described by Quastel and Bicki's (211) was used. Radioactivity in the nucleic acid fraction was determined according to Schneider's procedure (212).

After incubation, the manometer vessels were placed in crushed ice, the tissue was removed and washed twice with 10 ml ice-cold isotonic saline. The tissue was then homogenized in 95 per cent ethanol using a "Teflon" pestle homogenizer, and allowed to stand for 30 minutes. After centrifugation, an aliquot of the supernatant was plated on an aluminium planchet, dried and counted. Results were expressed as counts per minute per 100 mg. wet weight of tissue. In certain experiments in which the tissue was incubated with glycine-Cl, aliquots of the ethanol soluble fraction were spotted on Whatman No. 1 paper and two dimensional chromatography was carried out. Phase I contained secondary butanol, formic acid and water 70/11/17 (v/v/v). Phase II was composed of phenol, water and ammonia 960/110/3. Each phase was separately run overnight and allowed to dry for eight hours. About 90% of the radioactivity in the original spot was recovered as glycine.

The precipitate was washed once with ice-cold 6% TCA. 2 ml of 6% TCA were now added to the precipitate and the tubes were placed in a water bath at 90° for 20 minutes in order to hydrolyze the nucleic acids. The tubes were cooled and centrifuged. The supernatant was transferred to graduated centrifuge tubes. The residue was washed with 1 ml of 6% TCA. After centrifugation the supernatant was pooled with the first TCA extract. The TCA hydrolysate was extracted three
times with three volumes of ether, to remove the TCA, leaving an aqueous fraction, termed the nucleic acid fraction. Radioactivity was determined in aliquots of this fraction. Results were expressed as counts per minute per 100 mg. wet weight of tissue.

It was necessary to dispose of the exoskeleton prior to determining the specific activity of the protein. The precipitate was washed once with 5 ml of ether. The protein was then dissolved by adding 2 ml of 0.5 N KOH and allowed to stand for half an hour. The exoskeleton was separated by centrifugation. 0.2 ml of 70 per cent PCA was added to the precipitate the protein, which was then washed twice with 5 ml of 6 per cent TCA, once with 95 per cent ethanol. 5 ml ethanol-ether (3:1) were added and the tubes were placed in a 60°C water bath for 10 minutes. The ethanol-ether was removed by centrifugation. The protein was now washed once in ether, suspended in acetone and plated on weighed aluminium planchets. Results were expressed in terms of specific activity; counts per minute per mg. protein,

A further modification was required when whole insects were used for in-vivo studies, since these have a high lipid content (20 - 30% of the dry weight) (213). These were homogenized in 10 ml of 80 per cent ethanol and cooled in ice for half an hour. After centrifugation the ethanol was discarded and the precipitate was extracted with 5 ml of ethanol-ether 3:1 at 60°C for one hour (214). It was then washed twice with 5 ml ethanol-ether 3:1 and once with 5 ml acetone. The precipitate was dissolved in 2 ml of 0.5N KOH and allowed to stand for half an hour. The exoskeleton was removed by centrifugation
and the protein was precipitated. The nucleic acids were removed by the method of Schneider (212). The protein was suspended in acetone and plated on weighed aluminium planchets and measured as mentioned above.

**DETERMINATION OF RADIOACTIVITY IN THE ACID SOLUBLE FRACTION IN RNA AND DNA**

After incubation the tissues were removed and washed twice in ice-cold isotonic Krebs-Ringer solution. The tissues were homogenized in 5 ml ice-cold 6 per cent TCA and the homogenate was allowed to stand for 10 minutes in crushed ice. In the *in vivo* experiments the whole pupae were homogenized in ice cold 10 ml of 6 per cent TCA. After centrifugation the supernatant was extracted three times with three volumes of ether and the remaining aqueous fraction was termed the acid soluble fraction. Aliquots of this fraction were plated on aluminum planchets, dried and counted.

When adenine-8-C\textsuperscript{14} was used, the free bases and nucleosides were separated from the nucleotides by extracting the mixture 3 times with n-butanol saturated with water (215). This procedure removed free adenine-8-C\textsuperscript{14} and adenosine and left the nucleotides in the aqueous phase. Aliquots of each fraction were plated and counted.

Mono, di and triphosphonucleotides were separated by descending paper chromatography with a solvent mixture consisting of 75 parts 95 per cent ethanol and 30 parts 1 M ammonium acetate, pH 7.5 (216).

The acid insoluble residue was washed twice with 5 ml of 6 per cent TCA, once with 5 ml of 95 per cent ethanol, once with
5 ml ethanol-ether (3:1) and once with 5 ml ether. RNA was separated from DNA by the Schmidt-Thannhauser procedure (217). 2 ml of 0.5 N KOH were added to the insoluble residue and incubated overnight at 37°C to hydrolyze RNA. Then 0.2 ml of 70 per cent PCA was added and the tubes were kept in ice for 10 minutes. The supernatant which contained the RNA hydrolyzate was separated from the precipitate by centrifugation and transferred to graduated tubes. It was neutralized with KOH (phenol red was used as indicator). The potassium perchlorate was precipitated in the cold for 10 minutes. Aliquots of the RNA fraction were plated on aluminium planchets, dried and counted. Results were expressed as counts per minute per 100 mg tissue.

The perchloric acid precipitate which contained protein and DNA was washed twice with 5 ml of ice-cold 6 per cent TCA to remove potassium perchlorate. DNA was hydrolyzed with 2 ml TCA at 90°C for twenty minutes. After centrifugation the TCA was extracted three times with three volumes of ether. Aliquots of the DNA hydrolyzate were plated on aluminium planchets, dried and counted. Results were expressed as counts per minute per 100 mg wet weight of tissue. When the tissue was incubated with purine or pyrimidine bases or nucleosides, a modification of the method of Ellis and Scholefield for the isolation of DNA was used (218). The perchloric acid precipitate was washed twice with 5 ml of ice-cold 6 per cent TCA and once with ether. The precipitate was suspended in a small amount of acetone and was quantitatively plated on scratched aluminium planchets. It was dried and the radioactivity was determined. Results were expressed as already mentioned.
It has been reported (219), that the nucleic acid fraction of Ehrlich ascites cells prepared by the Schmidt-Thannhauser procedure is contaminated with radioactivity not associated with the nucleic acids but with phosphoprotein (220, 221). It was of interest to ascertain whether insect tissue would behave in the same manner. Ten first day pupae were injected with 2.5 µl glycine-1-Cl4 of specific activity 71.42 µc/mg. An aliquot of the RNA hydrolyzate was spotted on Whatman No. 3 chromatography paper. Electrophoresis was carried out for two hours in ammonium acetate buffer pH 3.4 at 1000 volts (222). More than 90 per cent of the original radioactivity was recovered in the 5-AMP and 5-GMP spots. This result is not surprising since the amount of phosphoprotein in tenebroid insects during metamorphosis is very low and is without variations (183). VARIATIONS IN THE EXTENT OF INCORPORATION OF RADIOACTIVE PRECURSORS

In the in vitro experiments the difference in radioactivity between duplicates did not exceed 10 per cent. On repeating the experiments the results did not vary by more than 15 per cent. In the in vivo experiments, however, each result represents an average value obtained from 4 to 8 insects, since there were individual differences in the extent of incorporation of radioactive substances into the various fractions examined. The results still remain highly significant since the differences in incorporation between the different groups varies from 50% to many fold.

DETERMINATION OF OPTICAL ROTATIONS

The determinations for optical rotation of solutions were carried out at room temperature with a Zeiss Photoelectric
Precision Polarimeter (±0.005°). Measurements were taken at wavelengths of 578 μm and 546 μm. Mathematical extrapolations to the sodium D line were carried out as follows:

\[
\alpha_{589°} = \frac{\alpha_{578°}}{\alpha_{546°} - \alpha_{578°}} \cdot \alpha_{546°}
\]

Results were expressed as the optical rotation at a wavelength of 589 μm (\(\alpha_{589°}\)).
OPTIMAL CONDITIONS FOR THE IN VITRO INCORPORATION OF PRECURSORS INTO VARIOUS CELL COMPONENTS

For mammalian tissues the conditions for in vitro incubations are well established and there are accepted (209) physiological solutions for this purpose. However, there is as yet no universally accepted physiological solution for incubating insect tissues. Buck (223) summarized a list of 19 different solutions which vary greatly in their composition. The concentration of NaCl ranges from 0.38 grammes per litre to 14.0 grammes per litre; that of KCl from 0.14 grammes per litre to 2 grammes per litre; that of CaCl$_2$ from 0.0 to 0.85 grammes per litre. The amounts of KH$_2$PO$_4$, MgSO$_4$ or MgCl$_2$ vary from 0.0-1.5, and 0-4 grammes per litre, respectively. These differences in the composition of the physiological solutions for incubation of insect tissues, are due to the fact that the concentration of ions in the blood varies tremendously in different species; and physiological solutions are designed to resemble the ionic composition of blood.

We, therefore, decided to test systematically the effect of ions and other factors on the in vitro incorporation of glycine-1-C$^{14}$ into various cell components. Most of these experiments were performed with first day pupae.

COMPARATIVE STUDY ON GLYCINE-1-C$^{14}$ INCORPORATION INTO PROTEIN AND NUCLEIC-ACIDS IN KREBS RINGER AND "TENEBRIO RINGER".

Preliminary experiments were carried out in Krebs-Ringer phosphate medium and in "Tenebrio medium". The latter medium was
composed in consideration of the concentration of ions reported for the blood of Tenebrio (224) and other coleopteran insects. The "Tenebrio medium" contained 86 mM NaCl, 40 mM KCl, 10 mM MgSO₄ and 5 mM CaCl₂. 10 mM glucose and 10 mM phosphate buffer pH 7.4 were added to both media. The tissues tested were dorsal abdominal slices and minced abdomens.

From Table 1 it is apparent that the extent of incorporation of glycine into protein and nucleic acids is similar in the two media as the Q₀₂ values. In both media the minced preparation incorporated glycine into protein, and nucleic acids at a lower level than did the slices. The Q₀₂ was also lower. This lower activity of the minced preparations is in agreement with reports concerning vertebrate tissues (225),

EFFECTS OF GLYCINE CONCENTRATIONS ON THE UPTAKE OF GLYCINE-1-C¹⁴ INTO PROTEIN AND NUCLEIC ACIDS.

Figures 1, 2 and 3 illustrate the results obtained when minced tissues from 1st day pupae were incubated in the presence of different concentrations of glycine, while the radioactivity per vessel remained constant.

The specific activity of the protein was approximately the same in samples incubated with 0.2 - 2.0 mM glycine. When higher concentrations of glycine were used there was a decrease in the specific activity of the protein, indicating isotopic dilution.

Determinations of incorporation of radioactivity into nucleic acids indicated that the system was saturated at a level of 0.2 mM glycine. Higher concentrations caused isotopic dilution.
TABLE 1.

GLYCINE-1-C14 INCORPORATION INTO PROTEIN AND NUCLEIC-ACIDS IN KREBS RINGER AND IN "TENEBRIO-RINGER"

(Protein expressed as counts per minute per mg., nucleic acids as counts per minute per 100 mg wet weight).

<table>
<thead>
<tr>
<th>KREBS RINGER</th>
<th>Minced Abdomen</th>
<th>Dorsal abdominal slice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QO2</td>
<td>Protein</td>
</tr>
<tr>
<td>1</td>
<td>4.2</td>
<td>280</td>
</tr>
<tr>
<td>2</td>
<td>3.6</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>5.3</td>
<td>305</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>200</td>
</tr>
</tbody>
</table>

| INSECT RINGER | | |
|---------------| | |
| 1             | 2.5 | 200 | 1,150 | 6.3 | 360 | 2,900 |
| 2             | 3.0 | 250 | 1,300 | 6.5 | 380 | 3,000 |
| 3             | 4.5 | 300 | 1,500 | 6.4 | 375 | 3,010 |
| 4             | 2.9 | 270 | 1,200 | 6.4 | 380 | 3,000 |

The tissues were incubated at 37°C for one hour in Krebs-Ringer phosphate buffer or in Tenebrio Ringer phosphate buffer pH 7.4 with a gas phase of oxygen, in the presence of 2mM glycine-1-C14 providing 400,000 cpm/μg per vessel. The final volume was 1 ml.
FIGURE 1

EFFECT OF GLYCINE CONCENTRATION ON THE UPTAKE OF GLYCINE-1-C\(^{14}\) INTO PROTEIN AND NUCLEIC ACIDS

Minced tissues of first day pupae were incubated at 37°C for one hour in Krebs-Ringer phosphate buffer pH 7.4. 10 mM glucose was present. Radioactivity per vessel was 400,000 counts per minute.
ABDOMEN

Experimental conditions as in Figure 1.
Experimental conditions as in Figure 1.
It was decided, therefore to incubate tissues in experiments to follow in the presence of 2 mM glycine, a concentration at which the system is saturated for both biochemical processes.

It is apparent from Figures 1 - 3 that in 1st day pupae incorporation of radioactivity into protein and nucleic acids is higher in the head than in the thorax or abdomen. These results indicate that there is greatest synthesis of protein and nucleic acids in the head. The extent of protein synthesis in the thorax is intermediate between that in the head and abdomen.

**EFFECT OF Na⁺ and K⁺ ON THE INCORPORATION OF GLYCINE INTO VARIOUS CELL COMPONENTS.**

The concentration of sodium and potassium ions in insect blood varies from species to species. In some species the concentration is close to that of human blood and in others it differs by a factor of 20 to 40 (224). The molar Na/K ratio in insect blood ranges from 25 to 0.1, the ratio being fairly constant for a given species. The phytophagous coleopteran insects of which *Tenebrio* is a member, were classified by Florkin (226) as a low-sodium-index class. *Tenebrio* blood contains 86 mM Na⁺ and 45 mM K⁺. In the low-sodium-index class the potassium index is always higher than in the high-sodium-index class. It was shown that a large shift in the dietary Na/K ratio induced only a minor shift in the blood ratio. (227) Recently, Harvey and Nedergaard (228) presented evidence that the midgut possesses a remarkable regulating mechanism.

The existence of insects with low Na/K ratios in the blood is of special interest from the standpoint of comparative physiology.
because the proper functioning of vertebrate muscle and nerve is usually thought to depend on a plasma Na/K considerably greater than 1. In man the Na/K ratio in blood serum is 29, while in Tenebrio it is 1:9. Insects, however, resemble man in having cells high in potassium rather than high in sodium. Roeder (229) and Griffiths and Tauber (230) reported that insect tissues are much more tolerant of the ionic environment than vertebrate tissues. They demonstrated the effects of various concentrations and ratios of potassium and calcium on spontaneous activity of nerve and crop in Periplaneta. Butz (231) showed that varying the Na/K ratio from 1.4 to 22.4 had no toxic effect on the heart beat of isolated hearts from larvae and pupae of Tenebrio. The K/Ca ratio could be varied from 0.3 to 3 and the osmotic pressure from 12.77 to 17.0, without any appreciable toxic effect.

When dorsal abdominal slices of first day pupae were incubated in the presence of different concentrations of sodium chloride it could be seen (Table 2) that sodium chloride concentrations ranging from 0 to 145 mM had no effect on respiration. In media containing low concentrations of sodium chloride (0-30 mM) and glycine-1-C\(^{14}\), the specific activity of the protein was forty per cent lower than in media containing 100-145 mM sodium chloride. These results were to be expected since Tenebrio blood contains 86 mM Na\(^+\).

The same was true for glycine-1-C\(^{14}\) incorporation into nucleic acids. In the presence of 100-145 mM sodium chloride the radioactivity was higher by 45 per cent than it was in media with a low concentration of sodium chloride (Table 2). It is of interest to note that even in
### TABLE 2

**EFFECT OF SODIUM CHLORIDE CONCENTRATIONS ON THE INCORPORATION OF C\(^{14}\) FROM GLYCINE-1-C\(^{14}\) INTO VARIOUS CELL COMPONENTS IN VITRO.**

<table>
<thead>
<tr>
<th>SODIUM CHLORIDE CONCENTRATION (mM)</th>
<th>0</th>
<th>30</th>
<th>100</th>
<th>145</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.3</td>
<td>6.2</td>
<td>6.3</td>
<td>6.4</td>
</tr>
<tr>
<td>QO(_2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (cpm/mg)</td>
<td>190</td>
<td>220</td>
<td>330</td>
<td>345</td>
</tr>
<tr>
<td>Nucleic Acids (cpm/100 mg tissue)*</td>
<td>1,500</td>
<td>1,750</td>
<td>2,890</td>
<td>3,000</td>
</tr>
<tr>
<td>Ethanol Soluble Fraction (cpm/100 mg tissue)*</td>
<td>19,500</td>
<td>19,000</td>
<td>21,500</td>
<td>19,500</td>
</tr>
<tr>
<td>BaCO(_3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cpm/100 mg tissue)*</td>
<td>390</td>
<td>350</td>
<td>375</td>
<td>380</td>
</tr>
</tbody>
</table>

*Wet weight of tissue*

Dorsal abdominal slices of first day pupae were incubated. Sodium chloride was replaced by sucrose to yield an ionic strength equivalent to 145 mM sodium chloride. Other conditions as in Fig. 1.
the absence of sodium ions glycine was incorporated into protein and nucleic acids to an appreciable extent. The amount of glycine incorporated was the same in the range of 0 - 30 mM sodium chloride.

The difference in the extent of incorporation of glycine into protein and nucleic acids in the presence of various concentrations of sodium chloride is not due to an effect of the ions on the transport of glycine into the cells. This is obvious from the estimations of radioactivity in the ethanol soluble fraction extracted from the tissue.

Varying the sodium chloride concentration did not alter the extent of oxidation of glycine as seen from Table 2. Glycine was oxidized at a very low level in the tissue examined.

In the experiments summarized in Table 2, where sodium chloride was replaced, sucrose was added to yield a final ionic strength equivalent to 145 mM sodium chloride.

In the serum of mammals about 66% of the total ionic strength of sodium, potassium, calcium and magnesium is balanced by chloride. In coleopteran insects chloride accounts for only 24% of the total cation titre and phosphate for about 3 per cent (223). In most other insects chloride and phosphate play a much smaller role in maintaining electroneutrality (7-17%). In Gastrophilus larvae chloride ions are responsible for 7% of the total cation titre. It was shown (146,232-234) that in these larvae that malate, fumarate, α-ketoglutarate, succinate and citrate account for 60% of the total cation titre.

From table 2 it is apparent that the best incorporation of glycine into various cell components is in the medium containing a high level of chloride ions.
Varying the amount of potassium in the medium had no effect on the $Q_{O_2}$ of dorsal abdominal slices (Table 3). The incorporation of glycine into protein and nucleic acids, as well as the uptake of glycine by the ethanol soluble fraction, were not influenced by altering the concentration of $K^+$ from 0 to 100 mM. Oxidation of glycine measured by the amount of radioactivity in $\text{BaCO}_3$, was lower by 48 per cent in samples incubated in the presence of 40 - 100 mM $K^+$. *Tenebrio* blood contains 40 mM $K^+$.

From the above it can, therefore, be concluded that the amounts of sodium and potassium ions yielding the best incorporation of glycine into various cell components and necessary for the oxidation of glycine in vitro, are similar to the amounts of these ions present in Krebs-Ringer solution.

**EFFECT OF Ca++ CONCENTRATIONS ON GLYCINE-1-Cl$^4$ INCORPORATION INTO PROTEIN NUCLEIC ACIDS AND THE ETHANOL SOLUBLE FRACTION OF DORSAL ABDOMINAL SLICES.**

In coleopteran insects Ca$^{++}$ is present in the blood at a level of 5 mM. The amount of calcium in insect blood is on the average higher than in vertebrates and lower than in other groups of invertebrates. The latter fact may be expected, since most insects do not harden their exoskeleton with calcium or secrete shells, as do other invertebrates. It has been shown that a change in the calcium content of the external medium has no effect on the function of axons and synapses in insects (229).

The presence of calcium ions in the medium is essential for the production of potassium contractions of isolated skeletal, heart and smooth muscle. Investigations of this nature with insect muscle have
### TABLE 3

**EFFECT OF K⁺ CONCENTRATIONS ON THE INCORPORATION OF GLYCINE-1-C¹⁴ INTO VARIOUS CELL COMPONENTS IN VITRO.**

<table>
<thead>
<tr>
<th>K⁺ CONCENTRATIONS (mM)</th>
<th>0</th>
<th>5</th>
<th>40</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q₀₂</td>
<td>6.2</td>
<td>6.3</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>Protein (cpm/mg)</td>
<td>275</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Nucleic Acids (cpm/100 mg tissue)*</td>
<td>2,750</td>
<td>2,750</td>
<td>2,750</td>
<td>2,800</td>
</tr>
<tr>
<td>Ethanol Soluble Fraction (cpm/100 mg tissue) *</td>
<td>19,150</td>
<td>18,750</td>
<td>19,500</td>
<td>19,500</td>
</tr>
<tr>
<td>BaCO₃ (cpm/100 mg tissue)*</td>
<td>325</td>
<td>345</td>
<td>180</td>
<td>172</td>
</tr>
</tbody>
</table>

* Wet Weight of tissue

Dorsal abdominal slices were incubated. The combined concentration of NaCl and KCl was 150 mM. 2 mM glycine-1-C¹⁴, 360,000 cpm per vessel were added. Other conditions as in Figure 1.
shown that the addition of calcium was not required. Maximal contractions took place in the absence of calcium. (235) It was recently shown, however, (236) that the isolated mesothoracic tibialis muscle of the locust *S. gregaria*, when placed in calcium free Ringer containing 4 mM disodium-ethylene-diamino tetra-acetic acid (EDTA), did not contract. Contractions occurred, when calcium ions were added to the medium. This experiment indicated that calcium ions are fixed inside the cells.

From Table 4 it is apparent that the addition of 5 mM Ca$$^{++}$$ to the medium in which dorsal abdominal slices were incubated, had no effect on the $Q_{O_2}$, on the incorporation of glycine-1-C$$^{14}$$ into protein, nucleic acids or the ethanol soluble fraction. The oxidation of glycine was also not affected by the presence of calcium ions.

**EFFECT OF MAGNESIUM IONS ON THE UPTAKE OF GLYCINE-1-C$$^{14}$$ BY CELL COMPONENTS.**

Magnesium ion concentrations range from 10 to 20 mM in the blood of coleopteran insects, while in vertebrate blood the concentration is about 1.5 mM. In marine invertebrates the magnesium content of the blood is still higher than it is in insects. It is assumed that the magnesium is derived from chlorophyll, though there is no clear correlation between diet and the blood magnesium content of the phytophagous insects studied.

The concentration of magnesium in insect blood is sufficiently high to induce anaesthesia in most non-marine animals.

Varying the Mg$$^{++}$$ concentration from 0 - 10 mM did not alter the incorporation of glycine-1-C$$^{14}$$ into protein nucleic acids or the ethanol soluble fraction of dorsal abdominal slices of first day pupae. The extent of oxidation of glycine-1-C$$^{14}$$ was the same for all Mg$$^{++}$$ concentrations. (Table 5).
### Table 4

**EFFECT OF Ca**++ **CONCENTRATIONS ON THE INCORPORATION OF GLYCINE-1-Cl**4 **INTO PROTEIN, NUCLEIC ACIDS AND THE ETHANOL SOLUBLE FRACTION IN VITRO**

<table>
<thead>
<tr>
<th>CONCENTRATION OF Ca**++** (mM)</th>
<th>0.0</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>QO2</td>
<td>6.5</td>
<td>6.6</td>
</tr>
<tr>
<td>Protein (cpm/mg)</td>
<td>325</td>
<td>330</td>
</tr>
<tr>
<td>Nucleic Acids*</td>
<td>2,600</td>
<td>2,550</td>
</tr>
<tr>
<td>Ethanol Soluble Fraction*</td>
<td>21,000</td>
<td>20,000</td>
</tr>
<tr>
<td>BaCO₃*</td>
<td>350</td>
<td>375</td>
</tr>
</tbody>
</table>

Dorsal abdominal slices of first day pupae were incubated. 2mM glycine containing 360,000 cpm/vessel were added. Other experimental conditions were as in **Fig. 1.**

*cpm/100mg wet weight tissue.
Table 5

EFFECT OF MG\(^{++}\) CONCENTRATIONS ON GLYCINE-1-C\(^{14}\) INCORPORATION INTO GLYCINE, NUCLEIC ACIDS AND THE ETHANOL SOLUBLE FRACTION IN VITRO

<table>
<thead>
<tr>
<th>CONCENTRATION OF Mg(^{++}) (mM)</th>
<th>0</th>
<th>1.5</th>
<th>5.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (cpm/mg)</td>
<td>280</td>
<td>300</td>
<td>320</td>
<td>330</td>
</tr>
<tr>
<td>Nucleic Acids*</td>
<td>2,500</td>
<td>2,750</td>
<td>2,600</td>
<td>2,750</td>
</tr>
<tr>
<td>Ethanol Soluble Fraction*</td>
<td>19,500</td>
<td>19,700</td>
<td>19,380</td>
<td>19,600</td>
</tr>
<tr>
<td>BaCO(_{3})*</td>
<td>330</td>
<td>350</td>
<td>360</td>
<td>340</td>
</tr>
</tbody>
</table>

Dorsal abdominal slices were incubated. 2 mM glycine-1-C\(^{14}\) containing 360,000 cpm were added. Experimental conditions as in Fig. 1.

*cpm/100 mg wet weight tissue.
EFFECT OF pH AND BUFFERS ON GLYCINE-1-C\textsubscript{14} INCORPORATION INTO PROTEIN AND NUCLEIC ACIDS.

Dorsal abdominal slices were incubated in Krebs-Ringer solution and the uptake of radioactivity from glycine-1-C\textsubscript{14} was determined in protein, nucleic acids and the ethanol soluble fraction over a pH range of 6.2 - 7.8. The values obtained over this range of pH were essentially the same as values attained at pH 7.4, which are represented in Table 6.

When no buffer was present in the medium the incorporation of radioactivity was the same as that achieved by the addition of 10 mM phosphate buffer. This result can be explained by the fact that no measurable glycolysis could be observed. (Table 6).

From Table 6 it can also be seen that the uptake of glycine into protein and nucleic acids was the same in the presence of bicarbonate and phosphate buffers pH 7.4.

EFFECT OF GLUCOSE AND ANAEROBIC CONDITIONS ON GLYCINE INCORPORATION INTO PROTEIN AND NUCLEIC ACIDS.

Minced tissue preparations of abdomen, thorax and head of first day pupae were incubated both aerobically and anaerobically, with and without glucose. The radioactivity incorporated from glycine-1-C\textsubscript{14} was determined in protein and nucleic acids. Under anaerobic conditions there was no uptake of radioactivity in the two fractions, (Table 7) suggesting that all the energy needed for biochemical processes involved, had to be derived from oxidative phosphorylation. In this respect insect tissues during metamorphosis differ from embryonic tissues to which they are often compared. Quastel and
**Table 6**

**EFFECT OF BUFFERS ON GLYCINE-1-C\(^{14}\) INCORPORATION INTO PROTEIN AND NUCLEIC ACIDS IN VITRO**

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Gas Phase</th>
<th>Protein (cpm/mg)</th>
<th>Nucleic Acids(^*)</th>
<th>BaCO(_3)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Phosphate Buffer pH 7.4</td>
<td>(O_2)</td>
<td>6.2</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>(CO_2)</td>
<td>---</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td>25mM Sodium Bicarbonate Buffer pH 7.4</td>
<td>Gas phase 5% CO(_2), 95% O(_2)</td>
<td>340</td>
<td>2,780</td>
<td>---</td>
</tr>
</tbody>
</table>

*Radioactivity in nucleic acids and BaCO\(_3\) and expressed as cpm/100mg wet weight of tissue. Dorsal abdominal slices of first day pupae were incubated. 2mM glycine-1-C\(^{14}\) were added containing 400,000 cpm. Other conditions as in "Fig. 1."
Table 7

**EFFECT OF GLUCOSE AND ANAEROBIC CONDITIONS ON GLYCINE-1-C\textsuperscript{14} INCORPORATION AND NUCLEIC ACIDS OF FIRST DAY PUPAE**

<table>
<thead>
<tr>
<th></th>
<th>Abdomen</th>
<th>Thorax</th>
<th>Head</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose mM</strong></td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><strong>Protein (cpm/mg)</strong></td>
<td>195</td>
<td>190</td>
<td>313</td>
</tr>
<tr>
<td><strong>Nucleic Acids (cpm/100mg wet weight of tissue)</strong></td>
<td>497</td>
<td>500</td>
<td>543</td>
</tr>
</tbody>
</table>

Minced first day pupae were incubated for one hour at 37°C in Krebs-Ringer phosphate medium for the aerobic experiment and in bicarbonate buffer for the anaerobic experiment. The media contained 2mM glycine-1-C\textsuperscript{14} (400,000 cpm/vessel). Other experimental conditions were as in 'Fig. 1.'
Bickis (211) showed that the highest efficiency ratio for glycine-\(^{14}\)C incorporation into protein, aerobically and anaerobically occurred in embryonic tissue. In the development of embryo to adult organs a marked drop takes place in the ability of the tissue to incorporate glycine into protein aerobically. Normal tissues having relatively high anaerobic glycolytic activities (for example kidney medulla, retina) may have little or no anaerobic efficiencies for the incorporation of glycine into their proteins.

The inability of insect tissues to incorporate glycine into protein and nucleic acids was observed throughout metamorphosis and also in adult tissues. The same is true also for the larval stage (Table 8). (Values for aerobic incorporation are given in the next chapter).

No measurable \(\text{O}_2\) was observed in the anaerobic experiments.

Addition of glucose did not increase the amount of glycine incorporated into protein and nucleic acids. This can be accounted for by the fact that R. Q. values for insect tissue during metamorphosis approach that for fat oxidation. Olson (237) showed that liver slices, display very slight aerobic and anaerobic glycolysis unaffected by the presence or absence of carbohydrate. In this tissue R.Q. values approach that for fat oxidation.

The inability to utilize anaerobic energy for protein and nucleic acid synthesis (Table 7) is in agreement with the observation of Bade and Wyatt (163), who investigated the utilization of fat and carbohydrate as a possible nutrient reserve in the prepupal
Table 8

EFFECT OF ANAEROBIC CONDITIONS ON GLYCINE-1-C\(^{14}\) INCORPORATION INTO PROTEIN AND NUCLEIC ACIDS OF LARVAE

<table>
<thead>
<tr>
<th></th>
<th>Abdomen</th>
<th>Thorax</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conditions</td>
<td>Protein (cpm/mg)</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Nucleic Acids (cpm/100mg wet weight of tissue)</td>
<td>102</td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conditions</td>
<td>Protein (cpm/mg)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Nucleic Acids (cpm/100mg wet weight of tissue)</td>
<td>0</td>
</tr>
</tbody>
</table>

10 mM glucose was present in the incubation medium other experimental conditions as in Table 7.
development of *cecropia* silkworm. With radio-isotopes they were able to show preferential utilization of fat as a nutrient, while the specific activity of glycogen remained constant. Bade, (166) by way of metabolic inhibitors, demonstrated that the operation of the citric acid cycle is the major pathway of acetate metabolism during metamorphosis. In view of these facts it is obvious that anaerobiosis will stop energy production and therefore protein and nucleic acid synthesis.

It can be seen in Tables 7 and 8 that much less glycine is incorporated into protein and nucleic acids of larval tissues than into first day pupal tissues.

In conclusion it may be said that the optimal conditions for glycine incorporation into protein and nucleic acids in our *in vitro* system, are met by Krebs-Ringer phosphate medium pH 7.4.

This medium was therefore chosen for *in vitro* incubations in the following experiments.
SUMMARY

1) The incorporation of glycine-1-c\(^{14}\) into protein and nucleic acids and the \(Q_{O_2}\) values in dorsal abdominal slices and minced preparations of first day pupae were similar in Krebs-Ringer and in "Tenebrio Medium". All values were higher in the slices.

2) 100 - 145 mM sodium chloride was the optimal range for uptake of radioactivity into protein and nucleic acids. \(Q_{O_2}\) values, oxidation of glycine and the incorporation of label into the ethanol soluble fraction were not affected in the range 0 - 145 mM sodium chloride.

3) \(K^+\) concentration ranging from 0 - 100 mM had no effect on glycine uptake into protein nucleic acids and the ethanol soluble fraction. Oxidation of glycine was inhibited in media containing a high concentration of \(K^+\).

4) \(Ca^{++}\) had no effect on incorporation of glycine into protein, nucleic acids or the ethanol soluble fraction, nor did it alter the oxidation of glycine.

5) 0 - 10 mM \(Mg^{++}\) in the medium did not influence the uptake of radioactivity into protein, nucleic acids or the ethanol soluble fraction.

6) Glycine metabolism was not altered in the pH range of 6.2 - 7.8. It was the same in the absence of buffer. Results did not differ in phosphate and bicarbonate buffers.

7) No measurable aerobic or anaerobic glycolysis could be observed.
8) The addition of glucose had no effect on respiration nor on glycine-1-\textsuperscript{14}C incorporation into protein and nucleic acids.

9) Oxygen is essential for glycine uptake by the latter fractions.

10) More glycine is incorporated in first day pupae than in larvae.
CHAPTER II

RESPIRATORY METABOLISM AND INCORPORATION OF PRECURSORS INTO PROTEIN AND NUCLEIC ACIDS

RESPIRATION

It is well established that during metamorphosis, in vivo respiration follows a U-shaped respiration curve. A U-shaped respiration curve for *Tenebrio* pupae was demonstrated by Krogh (71) and by Ludwig and Barsa (81).

Figure 4 shows a typical respiration curve for the pupal stage of *Tenebrio*. Weighed prepupae, pupae from first day to seventh day and first day adults were placed in Warburg flasks (5 animals per vessel). The gas phase was air and the temperature 28°C. Respiration was measured after twenty minutes thermal equilibration by the conventional manometric technique. Incubation was carried out without shaking to avoid reflex movements. After incubation the dry weight of the pupae was determined. The shape of the curve in Figure 4 is in full agreement with the curve demonstrated by Ludwig and Barsa (81) for *Tenebrio*. Their values were slightly higher, however, since they incubated the pupae at 30°C.

Since histolysis and histogenesis occur during metamorphosis, the U-shaped respiration curve could be due to the formation of new cell generations, which arise from larval cells. These cells are characterised by low respiration values. Their descendents are destined to give rise to further cell generations with high respiratory values characteristic of adult cells. The lower respiratory values are not
Animals were incubated at 28°C for 1 hour. Gas phase air.
due to a decrease in cell population, since the extent of histolysis is equivalent to that of histogenesis. The amount of DNA per pupa at any stage during the metamorphosis of Tenebrio is constant (125).

Another possibility for the course of respiration is that it may be due to an intracellularly controlled mechanism, which is not associated with cell division. If this is the case respiration should follow the same course in cells undergoing metamorphosis without division. We, therefore, measured respiration of the dorsal abdominal body wall slices. Wigglesworth (58) showed that this tissue differentiates into the adult tissue without cell division. As it will be shown later. DNA precursors are not incorporated into DNA in this tissue, indicating lack of DNA synthesis, which is to be expected.

In vitro respiratory values for dorsal abdominal body wall slices are given in Figure 5. It can be seen that respiration in this tissue follows a U-shaped curve. This phenomenon is most interesting, since in multicellular organisms, once cells have differentiated into a specific organ or form, the stimulation to produce more protein and nucleic acids, is associated with a rise in respiration. This is true for regenerating rat liver or during recovery from injury in many tissues. In Tenebrio pupae this is not the case, however. It will be seen here, that protein and nucleic acid synthesis are markedly increased with a simultaneous decrease in respiratory values for six of the seven days of pupation.

INTEGRATION OF C\(^{14}\) FROM GLYCINE-1-C\(^{14}\), FORMATE-C\(^{14}\) AND GLUCOSE-U-C\(^{14}\) INTO PROTEIN AND NUCLEIC ACIDS DURING METAMORPHOSIS

The last chapter dealt with the optimal conditions for glycine-
FIGURE 5

IN VITRO RESPIRATION CURVE OF THE DORSAL ABDOMINAL BODY WALL OF TENEBRIO PUPAE DURING METAMORPHOSIS

Tissue slices were incubated at 37°C for 1 hour in Krebs-Ringer phosphate buffer pH 7.4. 10 mM glucose were present. Gas phase was O2.
1-$^{14}$C incorporation into protein and nucleic acids. The fact that glycine is incorporated into nucleic acids suggests de-novo synthesis of purine bases. The determination of the precursors of uric acid in birds has often been cited as classic example of the use of radioisotopes in biochemistry. By administration of isotopes of carbon and nitrogen in various forms to pigeons it was established that glycine, serine, formate and carbon dioxide all provide fragments from which the uric acid molecule is formed. A variety of labelled compounds were fed to pigeons and the excreted uric acid was degraded to locate the position of the label. It was concluded that glycine is incorporated intact into purines to give rise to carbon 4 and 5 and to nitrogen 7; that carbons 2 and 8 come from formate or any other $C_1$ donor and carbon 6 from carbon dioxide (Fig. 6) (238). It was pointed out by Greenberg (239) that purines are synthesized as the corresponding ribotides and the first requirement in the de-novo synthesis of purines is the availability of ribose-5-phosphate for the synthesis of phosphoribosylpyrophosphate (PRPP) which is formed from hexoses.

Little is known of this pathway in insects. McEnroe and Forgash (240) injected $^{14}$C-formate into the American cockroach, and were able to demonstrate the incorporation of $^{14}$C into position 2 and 8 of the uric acid of the fat body. Of the $^{14}$C injected, 3-5% appeared in the uric acid of the fat body, some was eliminated as $^{14}$CO$_2$, very little was excreted unchanged and the remainder appeared as the 3 carbon of serine. It is therefore, likely that the pathway of uric acid synthesis in insects may be the same as that in birds, at least as far as carbons 2 and eight are concerned. The fact that glycine is incorporated into nucleic acids and all the counts are found in the
PRECURSORS OF THE PURINE RING

Aspartic acid

Glycine

Formate

Glutamine (amide)

Formate
adenylic and guanylic acid of RNA hydrolysate (see Materials and Methods), is further evidence that the pathway of purine synthesis in insects is similar to that reported for birds.

During larval life, glycine-1-C\textsuperscript{14} is incorporated into protein and nucleic acids \textit{in vitro} at a very low level as compared to pupae (Fig. 7, 8, 9). This fact can be explained by the slow growth of the larvae. Development from egg to pupa takes about four months at room temperature. (During larval ecdyses, however, high levels of glycine-1-C\textsuperscript{14} incorporation into protein have been observed). From the first day of pupation there was a sharp rise in glycine-1-C\textsuperscript{14} incorporation into protein in the abdomen (Fig. 7), which then remained constant with a slight decrease on the seventh day. This initial increase was low in the abdomen, higher in the thorax and most pronounced in the head (Fig. 7, 8, 9). In the head and thorax there was a steady rise in the specific activity of the protein. In adults the values of glycine-1-C\textsuperscript{14} incorporation into protein in the thorax and in the head, decreased to the larval values. In the abdomen the radioactivity was very high, probably because of spermatogenesis and oogenesis. Since the weight of the abdomen constitutes about 80% of the weight of the whole body, it was to be expected that the total values for the specific activity of the protein during pupation for the whole animal should remain constant.

This was found to be the case in the \textit{in vivo} experiments.
Minced insect tissues were incubated in Krebs-Ringer phosphate medium containing 10 mM glucose for 1 hr. at 37°C. Each flask contained 2 mM glycine-1-C\textsubscript{14}, 400,000 cpm. The values for larvae were measured at 4 different ages and found to be the same. The same was true for adults.
GLYCINE-1-C\textsuperscript{14} INCORPORATION INTO PROTEIN AND NUCLEIC ACIDS IN VITRO DURING METAMORPHOSIS

**Figure 7 & 8**

[Graph showing the incorporation of glycine-1-C\textsuperscript{14} into protein and nucleic acids in vitro during metamorphosis. The graph compares the levels of protein and nucleic acids in the abdomen and thorax across different stages of metamorphosis.]
FIGURE 9

GLYCINE-1-Cl4 INCORPORATION INTO PROTEIN AND NUCLEIC ACIDS IN VITRO DURING METAMORPHOSIS

HEAD

Experimental conditions as in Figure 7.
The amount of incorporation of glycine-1-C\textsuperscript{14} into protein \textit{in vivo} is given in Table 9. Pupae were injected with 2.5 \mu l of radioactive glycine (41,200 cpm, 2.2 \mu g/pupa) at intervals of 24 hours. The specific activity of the protein of the whole pupa was determined after incubation at 28°C for 24 hours. It is apparent from Table 9, that the rate of incorporation of glycine-1-C\textsuperscript{14} into protein does not change until the last day of pupation, when it drops by 50%. This drop in specific activity is probably not due to a decrease in protein synthesis. Since we measured the overall specific activity of proteins synthesized, this decrease in specific activity can be attributed to the synthesis of proteins which contain less glycine per unit weight. Under our experimental conditions the seventh day is the last day of the pupal stage, the adult emerging on the eighth day. The new adult cuticle is formed on the last day of the pupal stage and its major component is protein. Trim (241) has shown that the glycine content of insect cuticular protein is extremely low. The decrease in glycine incorporation per unit weight of protein, therefore, is not due to decreased protein synthesis; but reflects the synthesis of new proteins.

The incorporation of glycine into nucleic acids in the
### Table 9

**INCORPORATION OF GLYCINE-1-C\(^{14}\) INTO PROTEIN OF THE WHOLE PUPA IN VIVO**

<table>
<thead>
<tr>
<th>Day of pupation</th>
<th>Specific activity 3cpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>580</td>
</tr>
<tr>
<td>2</td>
<td>595</td>
</tr>
<tr>
<td>3</td>
<td>620</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
</tr>
<tr>
<td>5</td>
<td>590</td>
</tr>
<tr>
<td>6</td>
<td>610</td>
</tr>
<tr>
<td>7</td>
<td>300</td>
</tr>
</tbody>
</table>

Pupae from each day of pupation were injected with 2.5 \(\mu\)l of glycine-1-C\(^{14}\) (41,000 cpm, 2.2 \(\mu\)g/pupa). After 24 hours of incubation at 28\(^\circ\)C the specific activity of the protein was determined.
abdomen and in the thorax rose sharply from the beginning till the end of metamorphosis. A lag period of three days was observed in the head (Fig. 7-9). The highest values of glycine incorporation into nucleic acids were in the head and the lowest were in the abdomen. At the end of pupation the amount of glycine incorporated into nucleic acids in the head and thorax decreased to the initial larval values. In the abdomen, however, they remained high, most probably because of the activity of the gonads.

The pool of free glycine and the pool of nucleotides are constant during metamorphosis (125,197). The latter, which is very small is maintained from two sources, from cell breakdown and from de novo synthesis of nucleotides. Since the rate of histolysis and histogenesis does not change during metamorphosis, the increased uptake of glycine into nucleic acids, indicates an increase in the de novo synthesis of purine nucleotides, which keeps the pool constant.

Formate-C\textsuperscript{14} uptake by protein and nucleic acids and the oxidation of formate are shown in Table 10. First day pupae were injected with 10 μl sodium-formate-C\textsuperscript{14} (5.6 μg, 448,000 cpm per pupa) and incubated at 37°C for one hour. Radioactivity was then determined in the protein and nucleic acids of dorsal abdominal wall slices. The incorporation of formate into nucleic acids is further evidence for the de novo synthesis of purine and pyrimidine nucleotides in the pupae since formate is incorporated into thymine; a reaction catalyzed by a tetrahydrofolic-acid dependent enzyme. Incorporation of formate into protein indicates active metabolism of C\textsubscript{4} units. These results are in agreement with those obtained by McEnroe
Table 10

FORMATE - C\textsuperscript{14} INCORPORATION INTO PROTEIN AND NUCLEIC ACIDS IN VIVO

<table>
<thead>
<tr>
<th>No. of pupa</th>
<th>*Nucleic Acids (cpm/mg)</th>
<th>Protein (cpm/mg)</th>
<th>*C\textsuperscript{14}O\textsubscript{2} (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11,700</td>
<td>870</td>
<td>20,000</td>
</tr>
<tr>
<td>2</td>
<td>13,500</td>
<td>980</td>
<td>23,400</td>
</tr>
<tr>
<td>3</td>
<td>9,150</td>
<td>780</td>
<td>19,900</td>
</tr>
<tr>
<td>4</td>
<td>9,850</td>
<td>870</td>
<td>18,500</td>
</tr>
<tr>
<td>5</td>
<td>11,700</td>
<td>900</td>
<td>21,300</td>
</tr>
</tbody>
</table>

Mean 11,180 880 20,620

1st day pupae were injected with 10 \textsuperscript{ml} sodium-formate-C\textsuperscript{14} (5.6 \textsuperscript{mg}, 448,000 cpm per pupa) and incubated for 60 minutes at 37\textdegree C in Warburg flasks. Radioactivity in protein and nucleic acids was determined in dorsal abdominal slices. C\textsuperscript{14}O\textsubscript{2} was determined for whole pupae.

* cpmp/100 mg. wet weight tissue.
and Forgash (240), who examined the uptake of formate into protein and nucleic acids in the American cockroach, which is a hemimetabolous insect (an insect which does not undergo metamorphosis).

Further evidence for de novo synthesis of nucleotides in Tenebrio pupae is the incorporation of C\textsuperscript{14} from glucose-U-C\textsuperscript{14} into nucleic acids (Table 11). It is an established fact that most of the label from glucose found in the nucleotides and nucleic acids is in the ribose moiety. From Table 11, it can be seen that when 0.3 mM glucose was added, glucose was incorporated into protein. This occurs via the tri-carboxylic acid cycle and through transamination. 7% of the counts were found in the ethanol soluble fraction. At the concentration of glucose used, it was found to be well oxidized. By raising the concentration of glucose to 5 mM, 5% of the counts were found in the insect tissue (calculated to 100 mg wet weight). The C\textsuperscript{14}O\textsubscript{2} values fell to a negligible amount. Incorporation of radioactivity into the ethanol soluble fraction, however, remained high even with 5 mM glucose. These results indicate that glucose is not a good source of energy for the metabolic activities when the insect tissues are incubated in vitro. The reason for this is not because glucose is not taken up by the tissue. A comparison between the uptake of glucose by insect tissue and by brain cortex slices is shown in Table 11. The latter tissue was chosen as it is known to utilize energy from glucose oxidation. In brain cortex slices the ratio of C\textsuperscript{14} from glucose, found in the ethanol soluble fraction to C\textsuperscript{14} from oxidation of glucose \( \frac{C\textsuperscript{14}}{C\textsuperscript{14}O\textsubscript{2}} \) was 1.6 and in insect tissue the ratio was 83. These results indicate that brain cortex slices
Table 11

INTEGRATION OF C\textsuperscript{14} FROM GLUCOSE-U-C\textsuperscript{14} INTO NUCLEIC ACIDS, PROTEIN AND THE ETHANOL SOLUBLE FRACTION OF CHOPPED INSECT ABDOMINAL INTEGUMENT AND BRAIN CORTEX SLICES, AND DETERMINATION OF C\textsuperscript{14}O\textsubscript{2} GIVEN OFF

<table>
<thead>
<tr>
<th></th>
<th>Insect abdominal integument</th>
<th>Brain cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM Glucose</td>
<td>0.3</td>
<td>5.0</td>
</tr>
<tr>
<td>*Nucleic acids</td>
<td>1,800</td>
<td>---</td>
</tr>
<tr>
<td>Protein (cpm/mg)</td>
<td>294</td>
<td>---</td>
</tr>
<tr>
<td>*Ethanol soluble fraction</td>
<td>35,000</td>
<td>25,000</td>
</tr>
<tr>
<td>*C\textsuperscript{14}O\textsubscript{2}</td>
<td>10,500</td>
<td>300</td>
</tr>
<tr>
<td>*Ethanol soluble fraction/C\textsuperscript{14}O\textsubscript{2}</td>
<td>83</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Incubations were carried out in Krebs-Ringer phosphate medium pH 7.4 at 37°C for 1 hour. Each vessel contained glucose-U-C\textsuperscript{14} 500,000 cpm/vessel. Total volume was 1 ml. Gas phase was O\textsubscript{2}.

*Expressed as cpm/100mg wet weight tissue.
oxidize 52 times more glucose from the available intracellular pool, than does insect tissue. Bade and Wyatt (163), arrived at the same conclusion by a different experimental approach, in early pupae of cecropia. They did not measure the actual oxidation of glucose, but showed the incorporation of C^{14} from glucose into glycogen in vivo. The specific activity of the glycogen remained constant; the loss of body weight being at the expense of fats.

Since C^{14} from glucose is incorporated into nucleic acids, it is apparent that glucose is converted to ribose-5-phosphate, which is essential for the de-novo synthesis of purine and pyrimidine nucleotides. Two pathways are known by which hexoses are converted to ribose-5-phosphate:

1) the oxidative decarboxylation of glucose-6-phosphate, and

2) the conversion of hexose phosphates and triose phosphates to pentose phosphates, which is catalyzed by transketolase and transaldolase. Both pathways have been shown to occur in eggs, larvae and in adult insects (242-247), but not in pupae. The enzymes of the pentose cycle are located in the soluble fraction of the cell in insects as they are in other species.

The level of glucose-6-phosphate dehydrogenase serves to indicate the extent of activity of the shunt cycle (248). The enzymic activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of thorax homogenates of seventh day pupae, are given in Fig. 10. The enzymatic activities were assayed spectrophotometrically according to Glock and Mclean (249).
The reaction cuvette contained 600 μmoles MgCl₂, 30 μmoles TPN, 200 μmoles Tris buffer pH 7.4, 150 μmoles glucose-6-phosphate and homogenate as indicated above. Total volume was 3.0 ml. The reaction was run at room temperature and measurements were made in Bausch and Lomb automatic recorder spectrophotometer model 505. The tissue was prepared by homogenizing 7th day pupal thoraces (100 mg/ml/H₂O) for 1 minute in the cold and centrifuging at 3500 g for 10 minutes. The clear supernatant was used. The readings were made against a blank in which glucose-6-phosphate was omitted.

A - 1.0 ml supernatant
B - 0.5 ml supernatant
C - 0.2 ml supernatant
FIGURE 10

REDUCTION OF TPN
BY PUPAL THORAX HOMOGENATE
IN THE PRESENCE OF GLUCOSE-6-PHOSPHATE

Increase in optical density at 340nm

Time (sec.)
The reactions proceed as follows:

1) Glucose-6-phosphate + TPN $\xrightarrow{\text{Glucose-6-phosphate dehydrogenase}}$ 6-phosphogluconate + TPNH

2) 6-phosphogluconate + TPN $\xrightarrow{\text{6-phosphogluconate dehydrogenase}}$ ribulose-5-phosphate + CO$_2$ + TPNH

From Fig. 10 it is apparent that the hexose phosphate shunt operates in the pupae. Moreover, the reaction mixture after incubation gave a positive reaction with orcinol, indicating the formation of pentose.

INTEGRATION OF ADENINE-8-$^14C$ AND RADIOACTIVE NUCLEOSIDES INTO RNA AND DNA DURING PUPATION

Prior to the discovery of the de novo pathway for the biosynthesis of the purine ribonucleotides it was known that many microorganisms as well as higher organisms can utilize the purine bases and the purine ribonucleosides, although in the higher organisms, the utilization is very inefficient because of the rapid catabolism of these compounds, which appears to be more rapid in the case of guanine than in the case of adenine. Human cells grown in tissue culture are able to carry out de novo synthesis of purines but in the presence of aminopterin, an anti folic acid compound, this process is blocked and purines are required, showing that they can be used (250). Salzman and Sebring (251) have shown that HeLa cells in tissue culture can utilize both glycine and preformed adenine for purine nucleotides and that just as in micro-organisms, adenine prevents utilization of glycine.

The actual pathways for utilization of preformed bases involves three different enzymes, corresponding to the three reactions listed below using adenine as the purine:
1) Adenine + ribose-1-phosphate $\xrightarrow{\text{ribonucleoside \ phosphorylase}}$ adenosine + orthophosphate (252).

2) Adenine + PRPP $\xrightarrow{\text{ribonucleotide \ pyrophosphorylase}}$ adenosine-5'-phosphate + PP (253)

3) Adenosine + ATP $\xrightarrow{\text{ribonucleoside \ phosphokinase}}$ adenosine-5'-phosphate + ADP (254)

Reaction (2) can substitute for the combination of (1) and (3) as a means of converting adenine to AMP. It is generally thought that reaction (2) is the major pathway for converting purine bases into purine ribonucleotides. It is apparent that for the conversion of bases into ribonucleotides ATP is utilized directly, as in reaction (3) or indirectly for the formation of PRPP. Ribonucleoside phosphokinase (3) was found to act only on adenine ribosides and phosphokinase for the other bases was not found (254). Therefore it was thought to be less significant.

In Table 12 a comparison between glycine-1-C$^{14}$ and adenine-8-C$^{14}$ incorporation into nucleic acids in drosal abdominal slices of first day pupae is made. Though adenine is incorporated into RNA its utilization is inefficient in comparison to glycine, the latter being taken up about one hundred times more rapidly per $\mu$ mole atoms of C$^{14}$. Insect tissue resembles tissues from normal mammals and other higher organisms in this respect. The low efficiency for the incorporation of adenine into RNA is not due to lack of uptake of adenine into the acid soluble fraction. About 10 per cent of the amount added is found in this fraction.


Table 12

**UPTAKE OF GLYCINE-1-C\textsuperscript{14} AND ADENINE-8-C\textsuperscript{14} INTO RNA, DNA AND THE ACID SOLUBLE FRACTION IN VITRO**

(Values are given per 100 mg wet weight of tissue.)

<table>
<thead>
<tr>
<th>Glycine-1-C\textsuperscript{14}</th>
<th>RNA (cpm)</th>
<th>µmole atom C\textsuperscript{14}</th>
<th>DNA (cpm)</th>
<th>Acid soluble Fraction (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2mM 360,000 cpm/vessel</td>
<td>2,500</td>
<td>14</td>
<td>7</td>
<td>19,700</td>
</tr>
<tr>
<td>Adenine-8-C\textsuperscript{14}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02mM 222,000 &quot;</td>
<td>1,600</td>
<td>0.144</td>
<td>5</td>
<td>45,000</td>
</tr>
<tr>
<td>Adenine-8-C\textsuperscript{14}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3mM 380,000 &quot;</td>
<td>295</td>
<td>0.226</td>
<td>5</td>
<td>35,000</td>
</tr>
</tbody>
</table>

1st day pupae were used.

Dorsal abdominal slices were incubated.

Experimental conditions as in Table 11.
From Table 12 it is apparent that neither glycine nor adenine were incorporated into DNA in the dorsal abdominal slices of 1st day pupae, indicating absence of DNA synthesis. This was found to be the case throughout pupation, as can be seen in Table 13, and it is in accordance with Wigglesworth's observation (58) that no cell division takes place in this tissue during pupation in Tenebrio.

The stable rate of incorporation of radioactive adenine or uridine into the nucleic acid fraction in vitro was different from that observed for glycine either in vitro or in vivo, in which an increasing rate of incorporation was observed as pupation proceeded. These results may be due to the fact that the uptake of bases and nucleosides into nucleic acids is less efficient and after short time intervals is not significant. However, when the adenine and uridine were injected into the pupae and the latter were incubated in vivo for 24 hours, the incorporation into RNA in whole pupae, in dorsal and ventral body wall, was essentially the same as that of glycine in vitro (Fig. 11,13).

The incorporation of radioactive adenine and thymidine into DNA in the ventral and dorsal body walls during pupation are given in Fig. 11 and 13. The amount of adenine incorporated into DNA was about 50% of that incorporated into RNA.

EFFICIENCIES OF GLYCINE INCORPORATION INTO PROTEIN AND URIDINE INCORPORATION INTO RNA IN INSECT TISSUES DURING METAMORPHOSIS

Protein and nucleic acid synthesis were found to take place only under aerobic conditions during the larval stage and during metamorphosis (Table 7,8). It is well established that ATP is utilized
ADENINE-8-C\(^{14}\) INCORPORATION INTO RNA AND DNA IN VIVO IN DORSAL AND VENTRAL ABDOMINAL BODY WALL DURING METAMORPHOSIS

1st day pupae were injected with 10 \(\mu\)l adenine-8-C\(^{14}\) (220,000 cpm/pupa, specific activity 1.47 \(\mu\)c/mg). 1st sample was taken after 4 hours. Remaining samples were taken at 48 hour intervals.
FIGURE 12
INCORPORATION OF THYMIDINE-2-C\textsuperscript{14} INTO DNA OF DORSAL AND VENTRAL BODY WALL DURING METAMORPHOSIS IN VIVO

Thymidine-2-C\textsuperscript{14} (1.66 ug, 0.5 \mu c) was injected.

Other experimental conditions as in Figure 11.
FIGURE 13

Uridine-2-C\textsuperscript{14} (88,000 cpm, specific activity 122 \(\mu\)C per mg) in a volume of 2 \(\mu\)l was injected. Radioactivity in the RNA of whole pupae and of slices of dorsal abdominal body wall was determined after incubation for 24 hours at 28\(^\circ\)C.
FIGURE 13

INCORPORATION OF URIDINE-2-C\textsuperscript{14} INTO RNA OF DORSAL ABDOMINAL BODY WALL AND RNA OF THE WHOLE PUPAE IN VIVO DURING PUPATION

\[ x \times 10^3 \]

\[ cpm/100gm \text{ wet weight tissue} \]

\[ Dorsal \text{ abdominal body wall} \]

\[ Whole \text{ pupae} \]

Day of pupation

1 3 5 7
Table 13

INTEGRATION OF URIDINE-2-C\textsuperscript{14} AND ADENINE-8-C\textsuperscript{14} INTO RNA AND DNA IN VITRO

<table>
<thead>
<tr>
<th>Day of pupation</th>
<th>RNA</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine-2-C\textsuperscript{14}</td>
<td>RNA</td>
<td>2,340</td>
<td>2,250</td>
<td>2,080</td>
<td>3,000</td>
</tr>
<tr>
<td>357,000 cpm 6.5 µg/vessel</td>
<td>DNA</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Adenine-8-C\textsuperscript{14}</td>
<td>RNA</td>
<td>1,600</td>
<td>1,650</td>
<td>1,700</td>
<td>1,750</td>
</tr>
<tr>
<td>0.02mM 380,000 cpm/vessel</td>
<td>DNA</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

Experimental conditions as in Table 11.

Values express cpm/100 mg wet weight tissue.

Dorsal abdominal body wall slices incubated.
for the biosynthesis of protein and nucleic acids. In pupae, ATP is derived from oxidative phosphorylation, since the citric acid cycle is the major pathway for acetate metabolism (166). In considering the rate of incorporation of radio-isotopes into protein and nucleic acids during pupation as a general parameter for protein and nucleic acid synthesis, one can see that the rate of nucleic acid synthesis increases as pupation proceeds. (Figs. 7-9, 11-13). This was found in the whole body in vivo and in the dorsal abdominal wall, head thorax and abdomen in vitro. In the in vitro experiments the rate of protein synthesis increased in the head and thorax and remained constant for 6 of the 7 days of pupation in the abdomen. In the in vivo experiments the rate of protein synthesis was constant for 6 of the 7 days of pupation in the whole pupa.

An U-shaped curve for respiration was observed in vivo for the whole pupa and in vitro for the dorsal abdominal body wall slice during pupation (Figs. 4, 5). Since we found that during pupation energy can be utilized only under aerobic conditions, the above observation suggested that available ATP is utilized more efficiently for protein and nucleic acid synthesis in pupae than in larvae or in adults. This finding is of interest since in mammals and other species, a marked drop occurs in the ability of tissues to utilize ATP for protein synthesis as embryonic organs develop into adult forms (211).

The term "efficiency quotient", which was used to describe the ratio of amino-acid incorporation into protein to the ATP theoretically made available was first introduced by Quastel and Bickis (211). According to these authors if respiration is represented by the term
QO₂ then, taking P/O=3.0, the available ATP is given by Q_{ATP}=6QO₂. If the amount of incorporation of amino-acid into tissue protein is given by the term A in counts per minute per milligram dry weight protein, then the efficiency quotient E is equal to A/6QO₂.

The efficiency quotient has been adopted to examine the differences in the ratio of available ATP to rate of protein and nucleic acid biosynthesis during metamorphosis.

E for glycine-1-C₁⁴ incorporation into insect protein was calculated as cited above. E for incorporation of uridine-2-C₁⁴ into RNA was calculated from the quotient E_U = U/6 QO₂ when U is the amount of incorporation of uridine-2-C₁⁴ into insect tissue RNA per hour per 100 mg wet weight of tissue. The available ATP is given by the term Q_{ATP} = 6QO₂. since in insect mitochondria, as in mitochondria of other species, P/O ratio is approximately 3 (255,256).

Fig. 14 represents the extrapolation of the efficiency quotient for glycine-1-C₁⁴ incorporated in vitro into protein of the dorsal abdominal wall tissue during metamorphosis. It can be seen that the efficiency for glycine incorporation into protein is low in larvae, rises sharply in pupae, reaching a peak on the 5th day of pupation and then falls again to the initial larval value in the adult. In vivo (Fig. 15) in whole pupae the efficiency ratio is low on the first day of pupation, rises steadily, reaching a peak on the 5th day and then falls sharply on the last day. On the 7th day of pupation there is a drop in the rate of incorporation of glycine-1-C₁⁴ into tissue proteins. It has already been mentioned that this drop is probably not due to a drop in protein synthesis, but reflects a change of the
EFFICIENCY OF GLYCINE-1-\textsuperscript{14} INCORPORATION INTO PROTEIN OF DORSAL ABDOMINAL BODY WALL SLICES IN VITRO

The tissue was incubated at 37°C for 1 hour in Krebs-Ringer phosphate buffer pH 7.4 with a gas phase of O\textsubscript{2}, in the presence of 2 mM glycine-1-\textsuperscript{14} 360,000 cpm/vessel). Final volume 1 ml.
EFFICIENCY OF GLYCINE-1-C\textsuperscript{14} INCORPORATION INTO WHOLE PUPAL TISSUE PROTEIN IN VIVO

\[ Q_{\text{ATP}} \] was calculated from Figure 4.

Glycine-1-C\textsuperscript{14} incorporation into protein was taken from Table 9.
104.

Type of proteins synthesized. Protein synthesized at this stage contains less glycine per unit weight. (For experimental evidence see Chapter IV). The efficiency ratio was calculated on the assumption that no drop in synthesis of protein had occurred on the 7th day (Fig. 15 broken line). The curve obtained was essentially the same as that obtained from experimental values; there being a sharp drop in the efficiency ratio from the 5th to the last day of pupation. If Figs. 14 and 15 are compared qualitatively it can be seen that both in vivo and in vitro, there was a steady rise in the efficiency for glycine -1-C\(^{14}\) incorporation into protein, which reaches a peak on the 5th day and then falls sharply till the end of pupation.

The efficiency for uridine-2-C\(^{14}\) incorporation into RNA in slices of the dorsal abdominal wall in vitro, was low at the beginning of pupation, rising continuously and reaching a peak on the 5th day. From the 5th day it fell sharply till pupation terminated (Fig. 16). In vivo the efficiency curve follows a similar path (Fig. 17).

The same was also true when whole pupae were used for in vivo experiments (Fig. 18).

It can also be seen that the efficiency for uridine-2-C\(^{14}\) incorporation into RNA of dorsal abdominal body wall and whole pupae (Fig. 17 - 18) is similar on a quantitative basis.

A common feature for all the efficiency quotients expressed in this chapter, is the fact that their values are low at the beginning of pupation rising continuously, reaching maximal values on the 5th day and then dropping sharply to low levels at the end of pupation. In whole pupae this could be due to the fact that larval cells have
EFFICIENCY OF URIDINE-2-C\(^{14}\) INCORPORATION INTO RNA OF DORSAL ABDOMINAL BODY WALL SLICES OF PUPAE IN VITRO

\(Q_{ATP}\) was calculated from Fig. 5. Values for uridine-2-C\(^{14}\) incorporation were taken from Table 13.
FIGURE 17

EFFICIENCY OF URIDINE-2-C\textsuperscript{14} INCORPORATION INTO RNA OF THE DORSAL ABDOMINAL BODY WALL SLICES DURING PUPATION IN VIVO

Values for uridine incorporation were taken from Figure 13.

Values for Q\textsubscript{ATP} were calculated from Figure 4.
EFFICIENCY OF URIDINE-2-C\textsuperscript{14} INCORPORATION INTO RNA OF WHOLE PUPAE TISSUE DURING PUPATION IN VIVO

Values for uridine-2-C\textsuperscript{14} incorporation taken from Figure 13.

Values for $Q_{\text{ATP}}$ calculated from Figure 4.
low efficiency quotients, but that during metamorphosis new generations of cells arise, by division, possessing higher efficiencies than larval cells. The latter divide giving rise to adult cells with characteristic low efficiencies for synthesis of protein and nucleic acids. This explanation, however, is not valid in the case of a tissue such as the dorsal abdominal body wall, since no cell division occurs in this tissue. In this tissue, at least, the efficiency for utilizing available ATP must be controlled by some other mechanism, not associated with cell division.

Quastel and Bickis (211) have shown that normal adult tissues possess low efficiencies for incorporation of amino acids into protein in comparison to embryonic tissues. They pointed out that in the development of embryo into adult organs a marked drop takes place in the ability of the tissue to incorporate glycine into its proteins, although aerobically energy is apparently available in amounts comparable with those in the embryo. The holometabolous insect is a multiform organism in its post-embryonic life, when it alters its form three times, changing from larva to pupa to adult (more than three times in hypermetamorphotic insects). These forms are genetically controlled. During the pupal period larval tissues undergo reorganisation to adult tissues and organs which take on new functions and forms. Reorganisation of tissues in biological systems is accompanied by high levels of efficiency for protein and nucleic acid synthesis. This follows from the findings of Quastel and Bickis (211), since the vertebrate embryo is also a multiform organism, the different forms of which are genetically controlled.
Quastel and Bickis suggested that one possible cause for the diminished efficiency in adult tissues could be the development in the adult tissues of new enzyme systems dependent upon ATP, which are needed for the special functions of these tissues. Herrmann and Tootle (257) have made a survey of enzyme activities in developing tissues. The following are but few of the examples of liver enzymes which they quote: Tyrosine transaminase activity increases by more than 50% per day in the first four days of post-natal development. The activity of the transaminases tyrosine-ketoglutarate and phenylalanine-pyruvate increases by more than 50% per day from the 19th embryonic day till the first postnatal day. This is true for tryptophan pyrrolase between the 10th and 16th days of post-natal development. Histidase increases its activity from the 19th embryonic day to the 1st day of postnatal life by 50% a day.
SUMMARY

1) A U-shaped respiration curve was obtained when whole pupae and when tissue slices were incubated at 28°C.

2) A rise in the rate of incorporation of glycine-1-C\textsuperscript{14} into protein in the head and thorax was observed in vitro during metamorphosis; while in the abdomen the rate did not change till the last day of pupation and then dropped. The rate of incorporation of glycine was low in larvae and adult thorax but high in adult abdomen.

3) The rate of incorporation of radioactive glycine into protein in the whole pupa is steady in vivo till the last day of pupation and then drops.

4) There was a steady rise in incorporation of glycine-1-C\textsuperscript{14} into nucleic acids during metamorphosis in vitro, which drops in the adult in the head and thorax and remains high in the abdomen.

5) Formate-C\textsuperscript{14} was taken up into the nucleic acid fraction and protein of first day pupae.

6) Radioactive glucose was incorporated into protein and nucleic acids in vitro. At a concentration of 5 mM, the uptake of glucose into the ethanol soluble fraction was high, but the extent of its oxidation was negligible.

7) Brain cortex slices oxidize 52 fold more glucose from the intracellular available pool, than does insect tissue.

8) Glucose-6-phosphate dehydrogenase and 6-phosphogluconate activities were demonstrated in a homogenate of thorax from the 7th
day pupa. At the end of the incubation the mixture gave a positive orcinol reaction.

9) The rate of glycine incorporation into RNA in vitro was about 100 fold higher than that of adenine. This difference was not due to failure in uptake of adenine. Neither glycine nor adenine were incorporated into DNA of dorsal abdominal wall slices.

10) Radioactive adenine and uridine were incorporated into RNA in vitro at a steady rate during pupation. An increasing rate of incorporation into RNA was observed in vivo during pupation.

11) About 50% less adenine was incorporated into DNA than RNA in dorsal and ventral abdominal walls of pupae in vivo. Thymidine was incorporated into this tissue in vivo.

12) Efficiency quotients for glycine incorporation into protein in vivo and in vitro and for uridine incorporation into RNA in vivo and in vitro were low at the beginning of pupation, rising steadily reaching a peak on the 5th day and dropping to low levels at the end of pupation.
CHAPTER III

EFFECT OF COLCHICINE ON NUCLEIC ACID METABOLISM DURING METAMORPHOSIS.

Colchicine is a classical mitotic inhibitor and has remained a general standard of comparison for all antimitotic drugs because it inhibits at extremely low concentrations and is effective, either in vitro or in vivo or both in most plant and animal cells. The inhibitory effect on cell division has been demonstrated in insects (258). Voget (259) observed that treatment with colchicine changed the post embryonic differentiation of insect antennae. Moreover, it has been reported that treatment of the 5th larval instar (last) of *Bombyx* with colchicine brought about the development of giant pupae (260).

Although the effect of colchicine on mitosis has been more thoroughly studied than that of any other chemical there is a surprising lack of data on its biochemical mechanism of action. In fact its site of action has as yet not been defined. Lettre (261, 262) maintains that colchicine acts by preventing the utilization of ATP for chromosome movements. However, Baas (263) stated in his review on chemical influence on cell division, that there is not sufficient evidence for this conclusion. Furthermore, Amoore (264) showed that in certain tissues a profound drop in the level of ATP had relatively little effect on mitosis, a process which probably has only a small energy requirement.

It has been suggested by Swann (263) that nuclear RNA synthesis is modified, making it unavailable for release in adequate
concentration to participate effectively in the formation of the mitotic spindle. But it was shown by Taylor (266) and by Prescott and Bender (267), that the synthesis of protein and RNA are minimal during mitosis.

Benitez, Murray and Chargaff (268) reported that three diverse agents, mesoinositol, ATP and tropolone, would reverse the metaphase arrest of rat fibroblasts produced by colchicine. These authors found that when ATP was added to tissue cultures together with colchicine, there was a decrease in the degree of mitotic arrests. However, the percentage of cells in the various phases of division was the same in the presence of ATP as that found when no ATP was added.

Wang et al (269) found that treating rats in vivo with colchicine had little effect on the majority of nucleotides in the acid soluble pool of nucleotides in normal and in regenerating rat livers. They observed that colchicine increased the concentration of DPN and AMP and decreased the concentration of ATP, GTP and UTP.

Hell and Cox (270) showed using the radioautographic technique that colchicine depressed the number of grains in guinea-pig epidermal tissue which had been incubated with tritiated thymidine. This together with the facts that colchicine changed post embryonic differentiation in insects (259) and has been shown to be a mutagenic agent for coliform bacteria (271) and for the fungus Aspergillus spp (272); that colchicine increased the radio-sensitivity in the protozoan Paramecium (273) and caused malformation in the chick embryo (274), suggested that colchicine may act on nucleic acid metabolism.
THE BIOLOGICAL EFFECT OF COLCHICINE ON PUPAE OF TENEBRIO MOLITOR

Colchicine dissolved in sterile physiological saline was injected into first day pupae at concentrations ranging from 10 - 400 μg per pupa, in a volume of 10 μl. Control pupae were given injections of saline. The insects were incubated at 28°C, at which temperature pupation in the control group lasted 7 days, adults emerging on the eight day. Colchicine treated animals remained in the pupal stage for 2 to 3 weeks at the end of which they died without having matured. In pupae which had received 400 μg colchicine the mortality reached 30% within the two weeks. The criteria for deciding whether the pupae were alive were the following: a) reflex movement when touched, b) heartbeat and c) respiration.

EFFECT OF COLCHICINE ON THE INCORPORATION OF C\textsuperscript{14} FROM VARIOUS PRECURSORS INTO RNA AND DNA DURING PUPATION IN VIVO

First day pupae were injected with 10 μg colchicine and adenine-8-C\textsuperscript{14} and incubated at 28°C. Samples were removed after 4 hours and then at intervals of 48 hours for determination of incorporation of adenine into RNA and DNA in dorsal and ventral abdominal slices. In Fig. 19 it can be seen that on the third day there was a stimulation in the incorporation of adenine into RNA of pupae which had received colchicine. After the third day the amount of C\textsuperscript{14} decreased, indicating breakdown of tissue RNA.

The stimulatory effect of colchicine on the uptake of uridine by RNA in vivo is shown in Table 14. First day pupae were injected with colchicine and incubated at 28°C. Groups of insects were removed at 48 hour intervals, injected with radioactive uridine and incubated
FIGURE 19

EFFECT OF COLCHICINE ON ADENINE-8-C\(^{14}\) INTEGRATION INTO RNA IN VIVO

First day pupae were injected with 10\(^{-7}\) colchicine and adenine-8-C\(^{14}\) (specific activity 1.47 \(\mu\)c/mg) 220,000 cpm/pupa. Control pupae received saline and adenine. Samples of dorsal and ventral abdominal walls were taken 4 hours after injection. Remaining samples were taken at 48 hour intervals.
Table 14

EFFECT OF COLCHICINE ON URIDINE-2-C\textsuperscript{14} INCORPORATION INTO RNA OF DORSAL ABDOMINAL BODY WALL SLICES IN-VIVO

<table>
<thead>
<tr>
<th>Colchicine</th>
<th>Nil</th>
<th>200\mu g/pupa</th>
<th>400\mu g/pupa</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA (cpm/100 mg wet weight tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st day</td>
<td>12,660</td>
<td>14,000</td>
<td>9,870</td>
</tr>
<tr>
<td>3rd day</td>
<td>15,300</td>
<td>36,700</td>
<td>15,570</td>
</tr>
<tr>
<td>5th day</td>
<td>30,000</td>
<td>36,700</td>
<td>17,130</td>
</tr>
<tr>
<td>7th day</td>
<td>45,170</td>
<td>24,150</td>
<td>9,500</td>
</tr>
</tbody>
</table>

Colchicine was injected in a volume of 10 \mu l to first day pupae. Control insects received 10 \mu l of saline. Uridine-2-C\textsuperscript{14} (88,000 cpm/pupa) in 2 \mu l was injected on the first day and at 48 hour intervals. Pupae were incubated at 28\(^\circ\). Radioactivity in RNA was determined 24 hours after injection of uridine.
for 24 additional hours. The amount of radioactivity was then determined in RNA of whole pupae. With 200\textsuperscript{g} colchicine there was no effect on the first day. By the third day there was a stimulation, the counts in experimental pupae being 120\% higher than in controls. In pupae tested on the seventh day there was an inhibition of almost 50\% in the incorporation of uridine into RNA. 400\textsuperscript{g} colchicine caused an inhibition in RNA synthesis as from the first day.

10\textsuperscript{g} colchicine inhibited the incorporation of adenine into DNA. The effect was apparent as from the third day. By the fifth day the extent of inhibition in DNA synthesis was considerable (Fig. 20).

When thymidine-2-C\textsuperscript{14} was injected (Fig. 21) in the presence of 10\textsuperscript{g} colchicine, the inhibitory effect on uptake of thymidine by DNA was apparent after the third day of incubation. As from the fifth day the amount of radioactivity decreased, indicating breakdown of tissue DNA.

In all cases the first pronounced effect of colchicine on the incorporation of precursors into RNA or DNA was seen three days after the drug was injected. When colchicine is administered to laboratory animals, poisoning causes death several days later. Prior to death the animals suffer from severe gastro-intestinal disturbances and bone marrow depression (139).

The stimulation in RNA synthesis occurring on the third day, when DNA synthesis was inhibited, could have been due to the fact that the pool of free nucleotides was the limiting factor for these
FIGURE 20

EFFECT OF COLCHICINE ON ADENINE-8-C\textsuperscript{14} INCORPORATION INTO DNA IN VIVO

Conditions as in Figure 19.
FIGURE 21

EFFECT OF COLCHICINE ON THYMIDINE-2-\textsuperscript{14}C\textsubscript{14} INCORPORATION INTO DNA IN VIVO

[Graph showing the effect of colchicine on thymidine-2-\textsuperscript{14}C\textsubscript{14} incorporation into DNA.]

cpm/100mg wet weight tissue

1800
1200
600

\textsuperscript{14}C\textsubscript{14} Thymidine-2-

\textsuperscript{14}C\textsubscript{14} Thymidine-2-\textsuperscript{14}C\textsubscript{14} + 10 \textmu g Colchicine

Day of pupation
1 3 5 7
two biochemical processes, since both compete for free nucleotides. Nygaard et al. (275) studied the inter-relationship between RNA and DNA synthesis in the slime mould, Physarum polycephalum, as a model system for growth and differentiation. During synchronous growth RNA synthesis was retarded only at the time of DNA synthesis. The investigators explained this finding as a competition for common precursors for synthesis of DNA and RNA. A competition for common precursors for synthesis of DNA and RNA was shown by Grant (276) in the early developing embryo of Rana. He showed that DNA synthesis is regulated through the formation of thymidilic acid for which folic acid is a required co-factor and through competition of nucleotides for RNA synthesis. Folic acid analogs inhibited the conversion of deoxyuridine to deoxythymidine, thereby inhibiting DNA synthesis and simultaneously stimulating RNA synthesis. Sung and Quastel (277) reported a stimulation in vitro of adenine-8-C14 incorporation into RNA when DNA synthesis was inhibited by sarcomycin in Ehrlich ascites carcinoma cells.

EFFECT OF COLCHICINE ON ADENINE-8-C14 INCORPORATION INTO MAMMALIAN TISSUES IN VITRO.

Because of our findings concerning the effect of colchicine on insect tissues, we wished to ascertain whether we could arrive at similar results with mammalian tissues.

Table 15 shows the effect of colchicine on the uptake of adenine-8-C14 into DNA, RNA and the acid soluble fraction of Ehrlich ascites carcinoma cells, rat spleen, regenerating rat liver, rat embryo and guinea-pig mucosa, in vitro. In all mammalian tissues examined,
Table 15

EFFECT OF COLCHICINE ON ADENINE-8-C\textsuperscript{14} INCORPORATION INTO R N A, D N A AND THE ACID-SOLUBLE FRACTION IN MAMMALIAN TISSUES IN-VITRO

(Results expressed as cpm/100 mg wet weight for normal tissues and cpm/0.1 ml packed cells for ascites)

<table>
<thead>
<tr>
<th>Conc. of colchicine (mM)</th>
<th>Q\textsubscript{O2}</th>
<th>DNA cpm/100 mg wet wt. tissue</th>
<th>RNA cpm/100 mg wet wt. tissue</th>
<th>Acid Soluble Fraction cpm/100 mg wet wt. tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Ascites cells</td>
<td>6.9</td>
<td>6.7</td>
<td>4.2</td>
<td>428</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>69</td>
<td>67</td>
<td>42</td>
<td>428</td>
</tr>
<tr>
<td>Rat spleen</td>
<td>6.8</td>
<td>6.8</td>
<td>6.7</td>
<td>3.7</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>68</td>
<td>68</td>
<td>67</td>
<td>3.7</td>
</tr>
<tr>
<td>Regenerating rat liver</td>
<td>7.0</td>
<td>6.9</td>
<td>6.9</td>
<td>45</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>70</td>
<td>69</td>
<td>69</td>
<td>45</td>
</tr>
<tr>
<td>Rat embryo</td>
<td>2.9</td>
<td>2.9</td>
<td>2.9</td>
<td>2.5</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>2.5</td>
</tr>
<tr>
<td>Guinea-pig mucosa</td>
<td>16.2</td>
<td>16.0</td>
<td>16.4</td>
<td>8.8</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>8.8</td>
</tr>
</tbody>
</table>

* too low to count.

Tissues incubated in Krebs-Ringer phosphate buffer pH 7.4, with 10 mM glucose. Ascites cells were incubated with 1 mM adenine-8-C\textsuperscript{14}, 222,000 cpm/vessel. Normal tissues incubated with 0.1 mM adenine-8-C\textsuperscript{14}, 380,000 cpm. Total volume 3ml. Incubation was at 37°C for 1 hour. Gas phase for normal tissues was O\textsubscript{2} and for ascites cells air.
except for rat embryo, 10 mM colchicine had an inhibitory effect on O2 and on the uptake of adenine into the acid soluble fraction. /10 mM colchicine inhibited aerobic glycolysis by 22%. 1 mM colchicine had no effect on respiration and on the uptake of adenine into the acid soluble fraction, yet this concentration of drug inhibited the incorporation of adenine-8-C14 into DNA and to a slight extent, into RNA. 1 mM colchicine inhibited the incorporation of adenine-8-C14 into DNA of ascites cells by 51% and into RNA by 18.5%. In rat spleen incorporation into DNA and RNA were inhibited by 58% and 27.5% respectively. With rat embryo this concentration inhibited incorporation into DNA by 48% while there was no effect on RNA synthesis. The O2 and incorporation of adenine into RNA and the acid soluble fraction of rat embryo were not inhibited even with 10 mM colchicine. At the latter concentration uptake of radioactivity by DNA was inhibited by 76% in this tissue. With guinea pig mucosa 1 mM colchicine inhibited incorporation of adenine into DNA and RNA by 47.5% and 18% respectively.

In conclusion if we consider incorporation of adenine-8-C14 into RNA and DNA as a criterion for their synthesis then the results summarized in Table 15 indicate that 1 mM colchicine inhibited synthesis of DNA considerably and that of RNA to a slight degree. At this concentration of drug uptake of radioactivity by the acid soluble fraction and respiration were not affected.

One has to bear in mind that the lethal dose of colchicine is small for mammals being 5mg/Kg body weight for mice. With this dose, however, the toxic effect appears a week after administration. To obtain an in vitro effect the amount of drug was, therefore, raised.
EFFECT OF COLCHICINE ON THE INCORPORATION OF ADENINE-8-C\textsuperscript{14}, URIDINE-2-C\textsuperscript{14} AND GLUCOSE-U-C\textsuperscript{14} INTO RNA OF THE DORSAL ABDOMINAL WALL IN VITRO

In the biological experiments it was observed that 10\% colchicine per pupa inhibited the differentiation of pupa into adult. This result was not surprising since colchicine was known to be a mitotic inhibitor. However if lack of differentiation is due only to arrest of mitosis, a tissue such as pupal dorsal abdominal body wall should undergo differentiation since it does not undergo cell division. This is not the case, however. In this tissue therefore, other factors are responsible for the lack of differentiation.

When 200\% colchicine were injected into first day pupae (Table 14) and radioactive adenine was injected on the first, third, fifth and seventh days a 47\% inhibition in the rate of adenine incorporation into RNA was observed on the seventh day, indicating inhibition in RNA synthesis. (Incorporation was determined 24 hours after uridine injection). If RNA synthesis on the seventh day is responsible for differentiation of this tissue, then the inhibition in RNA synthesis could explain why differentiation had not occurred.

It is known that even ligated or isolated pupal abdomen can differentiate into the adult form. Gross and Cousineau (278) have shown that incorporation of labelled amino acids into proteins of fertilized sea-urchin eggs, continued for seven hours after synthesis of RNA (incorporation of uracil-C\textsuperscript{14}) was stopped by addition of a relatively high concentration of actinomycin. Under these conditions cell division continued but cell differentiation was suppressed. They claimed that stable messenger RNA for the formation of proteins
involved in cell division is present at fertilization and newly synthesized mRNA is needed only for the synthesis of relatively small quantities of "differentiation" proteins.

Since inhibition in RNA synthesis has been shown to arrest differentiation we decided to examine the effect of colchicine on RNA synthesis in the dorsal abdominal wall in vitro.

In Table 16 the effect of colchicine in vitro on adenine-8-C\(^{14}\) incorporation into RNA and the acid soluble fraction from the dorsal abdominal wall during pupation is given. 10 mM colchicine inhibited the incorporation of adenine into RNA by 42 - 75% during pupation. (10 mM colchicine in vitro corresponds to a dose of 400 \(\mu\)g in vivo, calculated on the pupal water content). 1 mM colchicine had no significant effect, however, from the third day of pupation it inhibited adenine incorporation into RNA to some extent. (Respiration was not affected by 10 mM colchicine). Neither the uptake of adenine by the acid soluble fraction nor the amount of nucleotide formed (counted after extraction with water saturated butanol) were inhibited by colchicine. Moreover, control samples of the acid soluble fraction were taken for paper chromatography. More than 80% of the radioactivity was found in ATP with 5 - 10% in ADP and only trace amounts of label were detected in AMP, adenine or adenosine. This result was not affected by the addition of colchicine (10 mM). This finding is contrary to what was found by Wang et al (269). They reported an increase in AMP and a decrease in the ATP content in normal and regenerating rat liver which had been treated with colchicine in vivo.
Table 16

**EFFECT OF COLCHICINE ON ADENINE-8-C\(^{14}\) INCORPORATION INTO RNA AND THE ACID SOLUBLE FRACTION IN DORSAL ABDOMINAL BODY WALL IN VITRO**

<table>
<thead>
<tr>
<th>Day of pupation</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine (mM)</td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

| RNA*            | 1,600 | 1,600 | 825  | 1,650 | 1,390 | 530  | 1,700 | 1,600 | 980  | 1,750 | 1,500 | 730  |
| % Inhibition    | 48    | 15    | 68   | 68    | 42    | 42   | 42    | 42    | 42   | 42    | 42    | 42   |

| Acid* Soluble Fraction | 45,000 | 45,000 | 45,000 | 44,000 | 45,000 | 45,000 | 41,000 | 43,000 | 42,000 | 43,000 | 42,000 | 45,000 |
| % Nucleotides (After extraction with water saturated butanol) | 92 | 96 | 91 | 95 | 92 | 94 | 90 | 89 | 89 | 90 | 89 | 92 |

Tissue was incubated in Krebs-Ringer phosphate buffer pH 7.4 at 37°C for 1 hour. 10 mM glucose and 18 umol adenine-8-C\(^{14}\) (222,000 cpm/ml) were present. Total volume 1 ml. Gas phase O\(_2\).
*Cpm/100 mg wet weight tissue.
Colchicine was tested for its effect \textit{in vitro} on adenine-8-C\textsuperscript{14}, uridine-2-C\textsuperscript{14} and glucose-U-C\textsuperscript{14} incorporation into RNA of dorsal abdominal body wall slices during metamorphosis. (Table 17). 10 mM colchicine inhibited the incorporation of adenine into RNA by 51 - 65\%, while 5 mM drug inhibited only by 29 - 37\%. The extent of inhibition of uptake of precursor was much higher with uridine and glucose. 10 mM colchicine inhibited the incorporation of uridine into tissue RNA by 83 - 88\% and of glucose by 77 - 81\%\footnote{5mM colchicine inhibited the incorporation of uridine into RNA by 57 - 71\% and that of glucose by 56 - 67\%. The reason for the different extents of inhibition on the incorporation of precursors used is not clear. It may be due to an exchange reaction between the labelled AMP moiety of ATP formed, with terminal adenylic acid moiety of the sRNA (C-C-A) molecules. The first report on such a reaction came from Heidelberger et al. (279).}

\textbf{TIME COURSE STUDIES}

A study of the effect of colchicine \textit{in vitro}, on the incorporation of adenine-8-C\textsuperscript{14}, uridine-2-C\textsuperscript{14} and glucose-U-C\textsuperscript{14} into RNA of dorsal abdominal body wall, as a function of time was made (Figs. 22 - 24). Incorporation of all three precursors both in the control and in the experimental pupae increased linearly with time for a period of two hours. There was no lag period in the inhibition caused by colchicine. The velocity of the enzymatic processes for adenine, uridine and glucose incorporation were inhibited by 50\%, 80\% and 74\% respectively in the presence of 10 mM colchicine. Again adenine incorporation into RNA was less inhibited than the incorporation of uridine or glucose.
Table 17

EFFECT OF COLCHICINE IN VITRO ON THE INCORPORATION OF ADENINE-8-C\textsuperscript{14}, URIDINE-2-C\textsuperscript{14} AND GLUCOSE-U-C\textsuperscript{14} INTO RNA OF DORSAL ABDOMINAL BODY WALL SLICES DURING PUPATION

<table>
<thead>
<tr>
<th>Day of pupation</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine (mM)</td>
<td>10</td>
<td>5</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Adenine-8-C\textsuperscript{14} (cpm/100 mg wet weight tissue)</td>
<td>1,090 (51)</td>
<td>1,600 (29)</td>
<td>2,245 (55)</td>
<td>1,750 (55)</td>
</tr>
<tr>
<td>Uridine-2-C\textsuperscript{14} (cpm/100 mg wet weight tissue)</td>
<td>218 (86)</td>
<td>552 (64)</td>
<td>1,560 (85)</td>
<td>232 (85)</td>
</tr>
<tr>
<td>Glucose-U-C\textsuperscript{14} (cpm/100 mg wet weight tissue)</td>
<td>585 (81)</td>
<td>1,040 (67)</td>
<td>3,080 (80)</td>
<td>577 (80)</td>
</tr>
</tbody>
</table>

Adenine-8-C\textsuperscript{14} - 400,000 cpm (16.8 ug) per vessel.

Uridine-2-C\textsuperscript{14} - 312,400 cpm (12.2 ug) per vessel.

Glucose-U-C\textsuperscript{14} - 524,600 cpm (28 ug) per vessel (carrier free).

Experimental conditions as in Table 16

Numbers in brackets represent per cent inhibition.
FIGURE 22

EFFECT OF COLCHICINE ON ADENINE-8-C\textsubscript{14} INCORPORATION INTO DORSAL ABDOMINAL BODY WALL SLICES IN VITRO AS A FUNCTION OF TIME

Tissue of first day pupae was incubated in Krebs-Ringer phosphate pH 7.4 in the presence of 10 mM glucose, adenine-8-C\textsubscript{14} 200,000 cpm (3.4 µg) and 10 mM colchicine. Final volume 1 ml. Gas phase O\textsubscript{2}. Temperature was 37°C.
EFFECT OF COLCHICINE ON URIDINE-2-C\textsuperscript{14}
INCORPORATION INTO DORSAL ABDOMINAL
BODY WALL SLICES IN VITRO
AS A FUNCTION OF TIME

Uridine-2-C\textsuperscript{14} 405,800 cpm/vessel (12 µg) were added as precursor.
Other conditions as in Fig. 22.
EFFECT OF COLCHICINE ON GLUCOSE-U-C\textsuperscript{14} INCORPORATION INTO DORSAL ABDOMINAL BODY WALL SLICES IN VITRO AS A FUNCTION OF TIME

Glucose-U-C\textsuperscript{14} $1.49 \times 10^6$ cpm/vessel (56 µg) with no glucose carrier, were added as precursor. Other experimental conditions as in Fig. 22.
Incorporation of uridine into RNA in vivo as a function of time, was followed in dorsal abdominal body wall slices (Fig. 25). The rate of inhibition was 70% in the first hour. As from the second hour there was a change in the velocity of incorporation in the control and in the colchicine treated pupae. Colchicine inhibited the velocity of the enzymatic activity by about 30%.

**EFFECT OF COLCHICINE ON GLYCINE-1-C^14 INCORPORATION INTO PROTEIN IN VIVO**

10 µl colchicine in 10 µl saline was injected into first day pupae. (Pupae were incubated at 28°C). At 24 hour intervals samples were injected with radioactive glycine (0.16 µg, specific activity 71.4 µg/mg) in a volume of 2.5 µl. The specific activity of protein in whole pupae was determined 24 hours after glycine had been injected. Colchicine did not interfere with the incorporation of glycine into protein during the first six days of pupation, the specific activity of protein being in the range of 540 - 570 cpm/mg in the control group as well as in samples.

25 µl, 50 µl and 100 µl colchicine in 10 µl and glycine (quantity as above) were injected to first day pupae. The specific activity of the protein was determined 24 hours later after the animals had been incubated at 28°C. Again the specific activity of the protein was not affected and was in the range mentioned above.

The results supply further evidence that the level of ATP is not affected by colchicine since ATP is required for protein synthesis.
4000× colchicine per pupa in a volume of 10 μl were injected into first day pupae.

Control pupae received 10 μl saline.

Uridine-2-C\textsuperscript{14}. 100,000 cpm per pupae in a volume of 2 μl were injected. Pupae were incubated at 28°C.
FIGURE 25
EFFECT OF COLCHICINE ON URIDINE-2-C\textsuperscript{14}
INCORPORATION INTO RNA OF DORSAL
ABDOMINAL BODY WALL IN VIVO
AS A FUNCTION OF TIME

17 000

10 000

5 000

0

Control

400 μ Colchicine

cpm/100gm wet weight tissue

Time (hours)

5 10 15 20 25
MODE OF ACTION OF COLCHICINE

We have shown that colchicine in low concentrations primarily inhibits DNA synthesis. The fact that compounds known to inhibit DNA synthesis act as mitotic inhibitors is well established (263). It is generally believed that polyfunctional alkylating agents which are mitotic inhibitors, primarily inhibit DNA synthesis. In E. coli sulfur mustard was found to inhibit the synthesis of DNA, and had little effect on RNA synthesis. There was little or no interference with the synthesis of acid soluble nucleotides (139). Folic acid derivatives which are mitotic inhibitors are thought to affect cell division through inhibition of DNA synthesis. HeLa cells grown in a medium containing adenine, when treated with methotrexate (folic acid antagonist), ceased to divide, but continued to increase in size. RNA and protein synthesis continued at a normal rate for 16 hours and then declined. Addition of thymidine for 16 to 20 hours caused the cells to recover and resume their normal proliferation rate (280).

The antibiotic mitomycin C which inhibits DNA synthesis is a mitotic inhibitor. It has been shown that it combines with the DNA molecule. Bacteria exposed to mitomycin C failed to utilize tritiated thymidine, and were shown to lose DNA presumably by breakdown of DNA (281).

Certain metaphase inhibitors which produce c-mitosis such as arsenic, heavy metals (mercury and cadmium) and iodo-acetate combine with the -SH group (282). Experiments were therefore carried out in order to determine whether cysteine and GSH would have a protective action on the inhibition by colchicine of adenine-8-C¹⁴ incorporation into nucleic acids. We found that neither 10 mM cysteine...
nor 10 mM GSH had a protective effect on the inhibition of adenine-8-C\textsuperscript{14} incorporation into DNA and RNA of Ehrlich ascites carcinoma cells by 1 mM colchicine. The same concentrations of cysteine and GSH did not neutralize the effect of 5 mM colchicine on uptake of adenine-8-C\textsuperscript{14} by RNA in dorsal abdominal body wall slices.

When Ehrlich ascites carcinoma cells were incubated with 1 mM colchicine in the presence of thymus DNA, the latter afforded full protection for incorporation of adenine into nucleic acids. When Ehrlich ascites carcinoma cells were incubated with 3 mM colchicine the incorporation of adenine-8-C\textsuperscript{14} into DNA and RNA was inhibited by 70% and 32% respectively (Table 18). When 2 mg DNA was added to the medium, inhibition by colchicine was almost completely abolished for uptake of adenine by DNA and was completely abolished for incorporation into RNA. 1 mM colchicine inhibited uptake of radioactivity by DNA and RNA by 51% and 17% respectively. There was no inhibition in the presence of 2 mg thymus DNA.

The results presented in Table 18 indicate, that addition of DNA to the medium protected incorporation of adenine into RNA and DNA in the presence of colchicine. This fact is suggestive of the possibility of combination of the colchicine with the DNA. In order to ascertain whether this is the case, the absorption spectra of DNA (10 \( \mu \)g/ml), of colchicine (3 \( \mu \)g/ml) and of a mixture containing the same concentration of each compound, were recorded against a blank of water (Fig. 26), using a Bausch and Lomb spectronic 505 recording spectrophotometer. The range scanned was that between 220 - 460 nm.
### Table 18

**EFFECT OF DNA ON THE INHIBITORY ACTION OF COLCHICINE ON ADENINE-8-C\(^{14}\) INCORPORATION INTO DNA AND RNA IN VITRO IN EHRLICH ASCITES CARCINOMA CELLS**

<table>
<thead>
<tr>
<th>Additions</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>630</td>
<td>11,600</td>
</tr>
<tr>
<td>DNA (2mg)</td>
<td>656</td>
<td>11,550</td>
</tr>
<tr>
<td>Colchicine 3mM</td>
<td>190 (70)</td>
<td>7,900 (32)</td>
</tr>
<tr>
<td>Colchicine 3mM + 2mg DNA</td>
<td>603</td>
<td>11,650</td>
</tr>
<tr>
<td>Colchicine 1mM</td>
<td>310 (51)</td>
<td>9,600 (17)</td>
</tr>
<tr>
<td>Colchicine 1mM + 2mg DNA</td>
<td>645</td>
<td>11,650</td>
</tr>
</tbody>
</table>

Numbers in brackets indicate % inhibition.

0.1 ml packed cells were incubated in Krebs-Ringer phosphate buffer pH 7.4 at 37°C for 1 hour in the presence of 10 mM glucose and 0.1mM adenine-8-C\(^{14}\), 380,000 cpm/vessel. Find volume 3 ml. Results expressed as cpm per 0.1ml packed cells. Gas phase air.
FIGURE 26

ABSORPTION SPECTRA OF DNA, COLCHICINE AND A MIXTURE OF DNA AND COLCHICINE

Dots represent sum of individual values for colchicine and DNA.
at room temperature. Colchicine exhibits maximal absorption at 245 μm and 354 μm, while DNA exhibits maximal absorption at 260 μm. The actual absorption spectrum of the mixture always differed slightly from the curve drawn by calculating the sum of the two separate absorption spectra for DNA and colchicine, in both downward slopes near the 245 μm and 260 μm regions. The absorbance of the mixture at 345 μm was always lower than the absorbance of colchicine and lower than the value obtained by calculating the sum of the separate absorbances of DNA and colchicine. If there is an interaction between the two substances it is difficult to demonstrate by this method since colchicine and DNA exhibit maximal absorption at 245 μm and at 260 μm, which are too close.

Though the above results were not significant they still indicated a possibility for the interaction of DNA with colchicine.

Since it is known that the optical rotation of colchicine in solution is strongly negative and that of DNA is strongly positive, we measured the optical activity of DNA and colchicine separately and as a mixture. Determinations were carried out at room temperature with a Zeiss, Photoelectric Precision Polarimeter. Results (Table 19) were expressed as the optical rotation at a wavelength of 589 μm (°589). The optical rotation of the mixture varied from the sum of the optical rotations of its components by 75% when the mixture contained 1 mg/ml DNA and 1 mg/ml colchicine. Keeping the concentration of DNA constant and taking lower concentrations of colchicine, with 0.5 mg colchicine per ml and 0.25 mg colchicine per ml the optical rotations of the mixtures varied from the sums of rotations of their
<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>Optical Rotation</th>
<th>Taking Colchicine as Zero</th>
<th>% Difference from Expected Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine 1mg/ml</td>
<td>-0.215°</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>+0.131°</td>
<td>+0.346</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA + Colchicine</td>
<td>-0.183°</td>
<td>+0.032</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Expected value</td>
<td>-0.084°</td>
<td>+0.131</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colchicine 0.5mg/ml</td>
<td>-0.107°</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>+0.138°</td>
<td>+0.245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA + Colchicine</td>
<td>-0.031°</td>
<td>+0.076</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Expected value</td>
<td>+0.031°</td>
<td>+0.138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colchicine 0.25mg/ml</td>
<td>-0.0492°</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>+0.138°</td>
<td>+0.1872</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA + Colchicine</td>
<td>+0.0483°</td>
<td>+0.097</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Expected value</td>
<td>+0.088°</td>
<td>+0.1372</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose 1mg/ml</td>
<td>-0.141°</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>+0.138°</td>
<td>+0.0279</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA + Fructose</td>
<td>+0.003°</td>
<td>+0.144</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Expected value</td>
<td>-0.003°</td>
<td>+0.138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose 0.5mg/ml</td>
<td>-0.0706°</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>+0.138°</td>
<td>+0.214</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA + Fructose</td>
<td>+0.063°</td>
<td>+0.1336</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Expected value</td>
<td>+0.067°</td>
<td>+0.1376</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Expected value is the sum of readings obtained when optical rotation of each compound of mixture is measured separately.
components by 45% and 29% respectively. As a control colchicine was replaced by fructose which has a negative optical rotation.

With fructose the percentage variation from calculated values for mixtures with DNA were 4% in the presence of 1 mg fructose per ml and 2.9% with 0.5 mg/ml fructose.

In conclusion, colchicine primarily inhibits DNA synthesis, while RNA is inhibited to a lesser extent. The presence of DNA in an incubation medium containing colchicine, protects Ehrlich ascites carcinoma cells from the inhibitory action of colchicine on the incorporation of adenine, into DNA and RNA.

Our experimental evidence strongly suggests that colchicine inhibits DNA synthesis by combining with DNA in a specific manner, thereby preventing DNA from serving as a primer (template) for DNA synthesis and to a slight degree for RNA synthesis.

A combination such as this is known to occur between mitomycin C and DNA. However, our experimental results do not rule out the possibility that the observed inhibition of incorporation of adenine-8-C\textsuperscript{14} and thymidine-2-C\textsuperscript{14} into DNA is not due to the inhibition of deoxycytidylic-, deoxyadenylic-, deoxyguanylic or deoxythmydic- kinases. Moreover, our observations do not eliminate the possibility of inhibition in formation of dexynucleotides or inhibition of DNA polymerase by combination with the enzyme.

Concentrations of colchicine which were used in our \textit{in vivo} and \textit{in vitro} experiments were much higher than those generally used for inhibition of mitosis. 2.5 \textmu M of colchicine when applied to isolated single grasshopper nerve cells during phophase, arrest metaphase
Cells at prophase are after the S period of the mitotic cycle and no DNA synthesis is taking place. During this period the cell already has twice the number of chromosomes. The mitotic inhibition is, therefore, not due to inhibition of DNA synthesis.

**EFFECT OF SARCOMYCIN ON MORPHOGENESIS**

Sung and Quastel (277) have shown that sarcomycin at a concentration of 100γ/ml (and higher), inhibited the rate of DNA synthesis by 90%. Only 20% inhibition of RNA synthesis was observed at a sarcomycin concentration of 100γ/ml. Almost no inhibition of adenine-8-C₁⁴ incorporation into acid-soluble nucleotides was observed with sarcomycin up to a concentration of 200γ/ml.

We compared the biological effect of sarcomycin with that of colchicine, on the differentiation of pupae into adult insects. 1st day pupae were injected with 60, 120 and 240γ sarcomycin dissolved in saline in a volume of 1 μl. Control animals were injected with saline. (30 pupae were injected with each concentration of drug). 240γ sarcomycin was lethal within one day to all pupae injected. 60 and 120γ drug completely arrested development, the pupae surviving for 2-3 weeks. The biological effect of sarcomycin was similar to that of colchicine.

**EFFECT OF MESCALINE ON MORPHOGENESIS AND INCORPORATION OF ADENINE INTO DNA AND RNA OF PUPAE**

The chemical formula of mescaline resembles part of the colchicine molecule (Fig. 27). Lettré (283), therefore, while searching for mitotic poisons, tried its effect on fibroblasts in tissue culture and found it to be devoid of action.
We injected 100\% mescaline (dissolved in saline, in a vol of 1 μl) into 40 pupae. The pupae survived for 2 - 3 weeks without undergoing any differentiation. Because of the similarity with the effect of colchicine we tested the action of mescaline on nucleic acid metabolism. 1st day pupae were injected with mescaline and adenine-8-C$^{14}$ and incubated at 28°C till their 5th day of pupation, when incorporation of radioactivity into RNA and DNA was measured. Results presented in Table 20 show that 160 and 80\% drug inhibited incorporation of precursor into DNA by 55% and 53% respectively. These concentrations were without effect on incorporation of adenine-8-C$^{14}$ into RNA, indicating that the drug did not interfere with energy metabolism.
COLCHICINE

MESCALINE
Table 20

EFFECT OF MESCALINE ON ADENINE-8-C\(^{14}\) INCORPORATION INTO RNA AND DNA OF WHOLE PUPAE IN VIVO

<table>
<thead>
<tr>
<th>Injections</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>6,050</td>
<td>37,000</td>
</tr>
<tr>
<td>80(\mu)mescaline/pupa</td>
<td>2,840 (53)</td>
<td>35,600</td>
</tr>
<tr>
<td>160(\mu)mescaline/pupa</td>
<td>2,720 (55)</td>
<td>41,000</td>
</tr>
</tbody>
</table>

First day pupae were injected with adenine-8-C\(^{14}\) (Specific activity 53 \(\mu\)c/mmole in a volume of 10 \(\mu\)l) and mescaline (1 \(\mu\)l). Control pupae were injected with saline. Radioactivity in DNA and RNA were determined after the pupae had been incubated for 5 days at 28\(^{\circ}\)C. Radioactivity was expressed as counts per minute per 100 mg wet weight tissue. Numbers in brackets represent % inhibition.
SUMMARY

1) Colchicine at concentrations of 10⁻⁴ - 400⁻⁴ /pupa arrested differentiation into the adult and prolonged the pupation period from 7 days to 2-3 weeks.

2) 10⁻⁴ colchicine inhibited adenine-8-C₁⁴ incorporation into DNA, stimulating incorporation into RNA, 3 days after administration of the drug to first day pupae.

3) Three days after administration of 10⁻⁴ colchicine, inhibition of thymidine-2-C₁⁴ incorporation into DNA was observed.

4) 200⁻⁴ colchicine stimulated uridine-2-C₁⁴ incorporation into RNA 3 days after administration of drug to first day pupae. At the end of pupation uridine-2-C₁⁴ incorporation was inhibited.

5) 5 - 10 mM colchicine did not inhibit the Q₀₂ nor uptake of radioactive adenine by the acid-soluble fraction in dorsal abdominal body wall slices in vitro. ATP formation was also not affected. These concentrations of drug inhibited incorporation of radioactive adenine, uridine and glucose into RNA.

6) The inhibitory effect of colchicine on uptake of radioactive precursors into RNA was more pronounced with uridine and glucose than with adenine.

7) The rate of incorporation of radioactive precursors into RNA in dorsal abdominal body wall in vitro was linear with time up to 2 hours. Colchicine inhibited the velocity of the enzymatic reaction. No lag was observed prior to inhibition. In vivo, the velocity of the enzymatic reaction for incorporation of uridine
into RNA was altered after 2 hours incubation in colchicine treated animals and in the control animals.

8) 1 mM colchicine inhibited the incorporation of radioactive adenine *in vitro* into DNA of mammalian tissues. Incorporation into RNA was only slightly inhibited. Respiration and uptake of radioactivity by the acid soluble fraction were not affected.

9) Thymus DNA protected adenine incorporation into DNA and RNA in Ehrlich ascites carcinoma cells, when the latter were incubated in the presence of colchicine.

10) Measurements of optical activity of DNA, colchicine and a mixture of both compounds indicated that combination of colchicine and DNA had occurred in solution.

11) Sarcomycin at a level of 60 and 120 µg/pupa completely arrested development of 1st day pupae, the latter surviving for 2-3 weeks.

12) 100 µg mescaline per pupa completely arrested differentiation of 1st day pupae. 80 and 160 µg drug inhibited incorporation of adenine-8-C<sup>14</sup> into DNA by 53 and 55% respectively. Incorporation into RNA was not affected.
Actinomycin D is a yellow peptide-containing antibiotic. The actinomycins are produced by Streptomyces species. These antibiotics were the first to be isolated from actinomyces and were reported by Waksman and Woodruff (284). They are, on the molar basis, the most potent antitumor substances known.

It was noted by Slotnik in 1958 that actinomycin D inhibited RNA, but not DNA synthesis in B. subtilis (285), and interfered with RNA synthesis in HeLa cells (286). Rounds et al (287) observed that the loss of nuclear RNA in cultures of conjunctival and HeLa cells treated with actinomycin represented an effect similar to that of ribonuclease. Actinomycin D, however, was shown to be strongly bonded to DNA but not to RNA in solution (288), and in bacteria it inhibited the activity of an enzyme fraction capable of incorporating deoxynucleotides into DNA. It was suggested that actinomycin D might interfere with the action of DNA polymerase (289).

Using C¹⁴ labelled actinomycin D, Harbers and Miller (290) found Ehrlich ascites cells took up the antibiotic rapidly; 80 per cent was found in the nucleus and 95 per cent of this was recovered from the DNA fraction. Raich et al (291) showed that actinomycin D inhibited the incorporation of tritiated cytidine into RNA, but did not interfere with tritiated thymidine incorporation into DNA of L cells in tissue culture. They further showed in strains L-929 and L-2 mouse fibroblasts that actinomycin D will completely uncouple the biosynthesis of normal cellular RNA from the biosynthesis of both DNA and protein for prolonged periods and that the synthesis
of RNA by these cells may be totally suppressed. The growth of the RNA containing Mengo virus is unaffected by actinomycin at concentrations higher than those which inhibit RNA synthesis by host cells, whereas vaccinia, a DNA containing virus is inhibited. The binding of actinomycin D to DNA is not irreversible since a DNA virus pre-incubated with actinomycin D may become viable when added to cells, presumably due to the competition of the cellular DNA for the viral bound actinomycin D (292). The ability to maintain protein synthesis after RNA production has been halted, suggested that the ribosomal informational template has considerable stability. Actinomycin D stopped all RNA production, indicating that cellular RNA formation is DNA dependent. In cell free systems, using bacterial (292) and mammalian (294) enzymes capable of catalyzing the DNA-dependent synthesis of RNA, actinomycin D inhibited the synthesis of polyribonucleotides. It was pointed out by Reich et al (292) that it seems probable that enzymes catalyzing DNA biosynthesis and DNA-dependent RNA biosynthesis differ significantly in their respective stereochemical relationship to the DNA model.

Kersten (295) showed that high concentrations of deoxyguanosine, and to a lesser extent guanine and adenine compounds, would alter the spectral properties of actinomycin D solutions. This has been confirmed (296). Bickis and Quastel (297) have shown that DNA is also active in protecting against the inhibitory effect of the antibiotic on adenine incorporation into RNA in Ehrlich ascites cells. Goldberg et al (298) have found in priming DNA dependent RNA synthesis, that the sensitivity to the antibiotic is a function of
G-C content of DNA primer; when DNA primers not containing deoxyguanosine residues are used, they do not bind actinomycin D (by spectral shift), nor is their priming of RNA synthesis inhibited.

In insects actinomycin D was used to investigate the puffs on the giant chromosomes of the salivary glands of Drosophila, which are known to be sites of active RNA synthesis. The puffs were suppressed by actinomycin (299). The antibiotic was also used to demonstrate the induction of DOPA-decarboxylase in prepupae of Calliphora (137).

**EFFECT OF ACTINOMYCIN D ON THE DIFFERENTIATION OF PUPA INTO ADULT**

Groups of 1st day pupae were injected with 1 μl of 0.04%, 0.08% and 0.16% actinomycin D per pupa. The control group which had received injections of saline and the group which had received 0.04% matured into normal adults after 7 days incubation at 28°C. In the group which had received 0.08% drug per pupa only 30% of the insects developed into normal adults. 0.16% actinomycin D per pupa modified adult development in all samples injected (40 animals in each group). Photographs of normal and modified Tenebrio beetles are given in Fig. 28. In the modified beetle, the head and thorax are essentially adult but the abdomen has pupal gin traps, urogomphi and pupal cuticle on the posterior segments. Wings did not develop. The modified adults were observed on the usual diet for three weeks. They exhibited normal movement but they did not lay eggs.

When 1 μl of 0.16% actinomycin D was injected into 1st, 2nd, 3rd, 4th, 5th, 6th and 7th day pupae (24 pupae per group), all
From left to right: Normal adult, modified adult and pupa.
pupae developed into modified adults, while control pupae developed into normal adults. These results indicated that actinomycin D when injected into 1st day pupae could not affect the normal development of the head and thorax. Moreover, injecting drug on the 7th day of pupation (last day) was sufficient to arrest development of the wings and abdomen.

**EFFECT OF ACTINOMYCIN D ON URIDINE-2-C\(^{14}\) INCORPORATION INTO RNA IN VIVO AND IN VITRO**

1st, 3rd, 5th and 7th day pupae were injected with 0.16\(\mu\) actinomycin D and radioactive uridine. After 24 hours incubation at 28°C (Fig. 29) it could be seen that the rate of uridine incorporation into RNA from whole pupae, increases with the day of pupation. 0.16\(\mu\) actinomycin D inhibited incorporation of uridine into RNA as from the 1st day by 51% and rose during pupation, to 67% on the 7th day.

In dorsal abdominal body wall (Fig. 30), 0.16\(\mu\) actinomycin D, inhibited incorporation of uridine into RNA by 51% on the 1st day rising to 75% on the 7th day.

When dorsal abdominal body wall slices of 1st day pupae were incubated in vitro with radioactive uridine and 1.6\(\mu\)/ml actinomycin D, incorporation of precursor was inhibited by 80%. In the presence of 0.80\(\mu\)and 0.40\(\mu\) drug per ml, uptake of uridine-2-C\(^{14}\) was inhibited by 65 and 32 per cent, respectively (Fig. 31).

The % inhibition was determined with the latter concentrations in 3rd, 5th and 7th day pupae and was found to increase with day of pupation (Table 21).
INCORPORATION OF URIDINE-2-C\textsuperscript{14} INTO RNA OF THE WHOLE BODY DURING PUPATION IN Vivo AND THE EFFECT OF ACTINOMYCIN D

0.16\,\mu\text{g} actinomycin D (vol 1\,\mu\text{l}) and uridine-2-C\textsuperscript{14} 88,000 cpm (specific activity 0.2\,\mu\text{Ci} per 12.6 mmoles vol 2\,\mu\text{l}) were injected to 1st, 3rd, 5th and 7th day pupae. Control insects received 1\,\mu\text{l} saline instead of actinomycin. Pupae were incubated for 24 hours at 28\,\degree C prior to determination of incorporation of radioactivity into RNA.
INCORPORATION OF URIDINE-2-\(^{14}\)C INTO RNA OF DORSAL ABDOMINAL BODY WALL DURING PUPATION IN VIVO AND THE EFFECT OF ACTINOMYCIN D

Experimental conditions as in Figure 29.
Dorsal abdominal body wall of 1st day pupae were incubated at 37°C for 1 hour in Krebs-Ringer phosphate medium pH 7.4 in the presence of 10 mM glucose, actinomycin D and uridine-2-C\textsuperscript{14} 405,000 cpm/vessel (6.53 μg). Total volume was 1 ml. Gas phase was oxygen. Per cent inhibition was calculated taking control value as 2240 cpm/100 mg wet weight of tissue.
# Table 21

**EFFECT OF ACTINOMYCIN D IN-VITRO ON URIDINE-2-C\textsubscript{14} INCORPORATION INTO R N A OF DORSAL ABDOMINAL BODY WALL DURING PUPATION**

<table>
<thead>
<tr>
<th>Day of Pupation</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Actinomycin</td>
<td>R N A cpm/100 mg wet weight tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2340</td>
<td>2520</td>
<td>2610</td>
<td>3000</td>
</tr>
<tr>
<td>0.4 μg/ml</td>
<td>1360 (41)</td>
<td>1350 (44)</td>
<td>1165 (55)</td>
<td>1160 (61)</td>
</tr>
<tr>
<td>0.8 μg/ml</td>
<td>820 (65)</td>
<td>800 (68)</td>
<td>765 (71)</td>
<td>780 (74)</td>
</tr>
</tbody>
</table>

Tissue was incubated for 1 hour at 37°C in Krebs-Ringer phosphate medium pH 7.4 in the presence of 10mM glucose and 6.5 μg uridine-2-C\textsubscript{14} 357,000 cpm/vessel. Gas phase was O\textsubscript{2}. Number in brackets represents % inhibition.
EFFECT OF THYMUS DNA ON THE INHIBITORY ACTION OF ACTINOMYCIN

Bickis and Quastel (297) were the first to show the protective effect of DNA on actinomycin inhibition of incorporation of radio isotopes into RNA in vitro. This has been confirmed by others (296). Goldberg and Rabinovitz (294) have shown a protective action of DNA on inhibition by actinomycin in extract of HeLa cells. This protective effect of DNA has not been shown in vivo, however.

The protective action of thymus DNA on the biological effect of actinomycin D as well as on the inhibition of uridine-2-C₁⁴ incorporation into RNA in vivo is demonstrated by results presented in Table 22. When 0.08% or 0.16% actinomycin D were injected into 6th day pupae the latter developed into modified adults. However, when this amount of drug was injected together with 80% DNA, normal adults developed. The same was true when 40% DNA were injected with 0.08% actinomycin. When DNA was injected first and the drug administered half an hour later, or vice versa, DNA failed to protect the inhibitory effect of actinomycin and modified adults were formed. When DNA was injected alone, normal adults emerged. Protection of the pupae from the inhibitory effect of actinomycin D occurred only when DNA and drug were injected together.

DNA was also found to protect incorporation of uridine-2-C₁⁴ into RNA in vivo when 40% DNA and 0.08% actinomycin were injected together (Table 22). When actinomycin D was injected alone, incorporation of uridine was inhibited by 63%, while no inhibition occurred when drug and DNA were mixed prior to injection. 38% inhibition occurred when DNA was injected half an hour before the drug and 41%
Table 22

PROTECTIVE ACTION OF THYMUS DNA ON INHIBITION OF MORPHOGENESIS AND INCORPORATION OF URIDINE-2-C\(^{14}\) INTO RNA BY ACTINOMYCIN D

<table>
<thead>
<tr>
<th>Substances injected</th>
<th>Biological Type of adult</th>
<th>Results Biochemical RNA cpm/100mg wet weight tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08(^{\gamma}) actinomycin D</td>
<td>Modified</td>
<td></td>
</tr>
<tr>
<td>0.16(^{\gamma})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.08(^{\gamma})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.16(^{\gamma})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80(^{\gamma}) DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 0.08(^{\gamma}) actinomycin D</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>+ 0.16(^{\gamma})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td>15,450</td>
</tr>
<tr>
<td>40(^{\gamma}) DNA</td>
<td></td>
<td>15,630</td>
</tr>
<tr>
<td>+ 0.08(^{\gamma})</td>
<td></td>
<td>14,000 (9)</td>
</tr>
<tr>
<td>+ 0.08(^{\gamma}) DNA</td>
<td>Modified</td>
<td>9,550 (38)</td>
</tr>
<tr>
<td>0.08(^{\gamma}) actinomycin D</td>
<td></td>
<td>9,088 (41)</td>
</tr>
<tr>
<td>+ saline</td>
<td></td>
<td>5,896 (63)</td>
</tr>
</tbody>
</table>

Total volume injected was 10\(\mu\)l. 6th day pupae were used.

In biochemical experiment uridine-2-C\(^{14}\) (specific activity 123\(\mu\)C/mg) 88,000 cpm/pupa was injected. RNA was determined after hours incubation at 28°C. Biological observations were made 48 hours after injections. Number in bracket represent percent inhibition.
inhibition when the order of injection was reversed. Again full protection by DNA only occurred when DNA and actinomycin D were mixed prior to injection. However, partial protection on the incorporation of uridine into RNA occurred also when DNA and drug were given separately. If inhibition of RNA synthesis (measured as uridine incorporation into RNA) is responsible for the modified morphogenesis, then 38% inhibition is sufficient to bring about the formation of a modified adult.

Thymus DNA had a protective effect on uridine-2-\(^{14}\)C incorporation into RNA when dorsal abdominal body wall slices were incubated \textit{in vitro} in the presence of 200\(\mu\)g and 400\(\mu\)g DNA per ml. and 0.8\(\mu\)g actinomycin per ml. (Table 23). When no DNA was present 0.8\(\mu\)g/ml actinomycin inhibited uptake of radioactivity by 70%.

The inhibition was 36% in the presence of 200\(\mu\)g DNA/ml, while 400\(\mu\)g DNA/ml gave full protection from inhibition.

The following experiment (Fig. 32) was carried out in an attempt to reverse the inhibitory action of actinomycin D on incorporation of uridine-2-\(^{14}\)C into RNA \textit{in vitro}. Dorsal abdominal body wall slices were incubated for 45 minutes then 0.8\(\mu\)g actinomycin (in 0.1 ml saline, control receiving saline) were added. 45 minutes later 400\(\mu\)g DNA or 400\(\mu\)g deoxyguanosine (in 0.1 ml saline) were added. Samples were taken every 45 minutes for determination of radioactivity in RNA. Both DNA and deoxyguanosine completely reversed the inhibition by actinomycin D.
Table 23

PROTECTIVE ACTION OF THYMUS DNA ON THE INHIBITORY EFFECT OF ACTINOMYCIN D ON THE INCORPORATION OF URIDINE-2-\textsuperscript{14} INTO RNA OF DORSAL ABDOMINAL BODY WALL IN VITRO

<table>
<thead>
<tr>
<th>Additions</th>
<th>RNA (cpm/100mg wet weight tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no addition)</td>
<td>2,500</td>
</tr>
<tr>
<td>0.8 actinomycin D/ml</td>
<td>880</td>
</tr>
<tr>
<td>200 DNA/ml + 0.8 actinomycin D/ml</td>
<td>1,600 (35)</td>
</tr>
<tr>
<td>400 DNA/ml + 0.8 actinomycin D/ml</td>
<td>2,400</td>
</tr>
<tr>
<td>400 DNA per ml</td>
<td>2,450</td>
</tr>
</tbody>
</table>

1st day pupae were used. Experimental conditions as in Table 21.
REVERSAL OF THE INHIBITORY ACTION OF ACTINOMYCIN D ON URIDINE-2-C\(^{14}\) INCORPORATION INTO RNA, BY DNA AND DEOXYGUANOSINE IN VITRO

Dorsal abdominal body wall slices of first day pupae were incubated in Krebs-Ringer phosphate medium pH 7.4, at 37°C for 135 minutes. 10 mM glucose and uridine-2-C\(^{14}\) 200,000 cpm (3.2 \(\mu\)g) were present. Initial volume 0.9 ml. Gas phase was oxygen. At point A 0.8\(\mu\)g actinomycin D in 0.1 ml saline or saline alone were added. At point B 400\(\mu\)g deoxyguanosine or 400\(\mu\)g DNA or saline (0.1 ml) were added.
ACTINOMYCIN D AND PROTEIN SYNTHESIS DURING PUPATION

Since it is generally believed that cellular RNA is produced by a DNA-dependent reaction, the use of the antibiotic might be expected to permit the demonstration of the stability of protein forming units. Jacob and Monod (300) made a suggestion that has been widely accepted, that RNA templates are short lived and require renewal. However, there are several examples of stable protein-synthesizing systems. These include a) enucleate amoebae, b) enucleate Acetabulariae, and c) mammalian reticulocytes (also enucleate). In these cases protein synthesis proceeds for prolonged periods, and the absence of a nucleus eliminates any source of genetic information which might provide a continuous supply of unstable "messengers". There are indications that even bacteria, the protein synthesizing units may be stable. When a very high concentration of actinomycin (which completely suppressed RNA synthesis) was added to B. cereus cultures following commitment to sporulation, there was no interference with the normal rate or extent of sporulation, and the viability and heat stability of the small spores produced was normal (301). This experiment shows that the complex morphogenetic process of sporulation, requiring for its completion many hours of protein synthesis and involving an orderly controlled, sequential induction of enzyme activities, could proceed normally in the complete absence of RNA synthesis. Hiatt (302) working with liver and regenerating rat liver and using C14 labelled orotic acid, found that the rapidly labelled RNA fraction which he identified as messenger RNA, is absent from cytoplasm. This finding led him to the conclusion
that renewable mRNA may exist in liver nuclei, and a relatively stable template in cytoplasm. Recently Revel and Hiatt (303) have shown that purified cytoplasmic RNA from control and actinomycin treated rats produced an equal stimulatory effect in an in vitro protein-synthesizing system derived from E. coli. Amino acid incorporation into liver protein in vivo was unaffected for as long as 40 hours following injection of the antibiotic; microsomal fractions from treated rats showed no impairment in in vitro amino acid incorporation studies; in liver nuclear RNA reduction of isotope incorporation by the antibiotic approximated the diminution in stimulatory activity of the purified nuclear RNA in in vitro amino acid incorporating systems.

However, there was no concomitant reduction of in vivo amino acid incorporation into liver nuclei of treated animals. The demonstration that a decrease in nuclear stimulatory activity, of the amino acid incorporating system, approximating the reduction in nuclear labelled RNA suggests that the level of actinomycin used (1.5 µg/gm), inhibits the messenger RNA. Revel and Hiatt came to the conclusion that the bulk of the cytoplasmic RNA fraction is stable for at least 40 hours.

In our experiments we used a similar concentration of actinomycin (0.16 µg/120 mg wet weight tissue), which inhibited 51-67 per cent of the incorporation of labelled uridine into RNA (Fig. 29). Messenger RNA is the most rapidly labelled RNA, 19% inhibition of total RNA is sufficient to inhibit mRNA production in liver nuclei (303). Levinthal et al (304) have shown, using
B. aubtilis, which is susceptible to actinomycin, that in this organism, the half life of mRNA is 2 minutes. Cessation of protein synthesis arises exclusively from interruption of RNA synthesis. When valine-$^{14}C$ was mixed with actinomycin D, the $^{14}C$ incorporation into protein continued for a short time (3 minutes) at the same rate as the control and then decreased rapidly. The total amount incorporated in 15 minutes was equal to the amount incorporated by the control in 4 minutes. However, Acs et al (305) have shown that in this system, newly-formed RNA of high molecular weight is rapidly degraded in bacteria exposed to actinomycin. This depolymerization is a direct effect of the antibiotic and not simply a consequence of inhibition of RNA synthesis.

Chloramphenicol RNA (RNA formed in the presence of chloramphenicol) is normally transferred intact into stable ribosomal form, without intervening turnover. In isolated thymus nuclei the protein synthesizing system is not stable. In this system the presence of actinomycin D inhibited 46% of labelled uridine incorporation into RNA (the inhibition is immediate in the presence of the drug). However, alanine-$^1$C incorporation into protein in the presence of the same concentration of the drug continued for 60 minutes and then showed a progressive inhibition. This finding was explained by the fact that mRNAs required for protein synthesis gradually "turned over" and needed to be replaced (42).

We studied the effect of actinomycin D on glycine-$^{14}C$ incorporation into protein during pupation. The drug was injected into 1st day pupae which were then incubated at $28^°C$. Groups were removed from the incubator at 24 hour intervals injected with radioactive
glycine and replaced in the incubator. Incorporation of glycine into protein was determined 24 hours later. The specific activity of the protein (Table 24) was the same in the experimental groups and in the control group for the first 6 days of pupation. In the control group injected on the 7th day, the specific activity of the protein was half that observed on previous days. From the time the glycine was injected to the time when the specific activity was determined the pupae had become adults. The specific activity of protein in the experimental group did not show this drop. These pupae developed into modified adults. It may be that during pupation the protein synthesized has the same glycine content per unit weight, until the 7th day when a new type of protein is synthesized. This is probably due to the formation of new cuticle and the development of wings. In modified adults this shift was not observed.

This experiment suggests that the protein synthesizing template is stable during 6 of the 7 days of metamorphosis; that on the 7th day there is a qualitative change in the protein synthesized on this template. This change is sensitive to actinomycin D and under the influence of the drug the "pupal protein synthesizing system" remains active on the 7th day. This means that the messenger RNAs formed during pupation are stable for 6 of the 7 days of pupation. On the 7th day these messengers are replaced by new messengers which produce new types of protein. When the production of these new mRNAs is blocked by actinomycin D, the template remains stable. Essentially the same results were obtained when the amino acid and the drug were injected together on each day of pupation. Again a 50% drop was observed in control animals injected
Table 24

EFFECT OF ACTINOMYCIN D ON GLYCINE-1-\textsuperscript{14}C INCORPORATION INTO PROTEIN
OF WHOLE PUPAE IN VIVO

<table>
<thead>
<tr>
<th>Day of pupation</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>580</td>
<td>575</td>
</tr>
<tr>
<td>2</td>
<td>595</td>
<td>600</td>
</tr>
<tr>
<td>3</td>
<td>620</td>
<td>610</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
<td>615</td>
</tr>
<tr>
<td>5</td>
<td>590</td>
<td>595</td>
</tr>
<tr>
<td>6</td>
<td>610</td>
<td>615</td>
</tr>
<tr>
<td>7</td>
<td>300</td>
<td>610</td>
</tr>
</tbody>
</table>

1st day pupae were injected with 0.16\% actinomycin D in a volume of 1 µl. Control pupae were injected with saline. Other experimental conditions as in Table 9.
on the 7th day of pupation.

Actinomycin D can alter the course of RNA synthesis and morphogenesis even when it is administered to 7th day pupae. Actinomycin D prevents the drop in specific activity observed in normal 7th day pupae.

**FARNESOL AND METAMORPHOSIS**

Farnesol is known to mimic the action of juvenile hormone (53). Certain derivatives of farnesol are much more active than farnesol (56). Many terpenoids and alcohols have been tested for juvenile hormone activity, using farnesol as an index material. Many materials have been assayed by the *Tenebrio* test, the criterion for the test being whether materials injected into 24 hour old pupae cause the latter to retain pupal characters. We compared the modified adults we obtained by means of actinomycin with the results obtained by Bowers and Thompson (306) with farnesol, its derivatives, isoprenoids and straight-chain alcohols. Our modified adults looked identical to those obtained by Bowers and Thompson. We therefore repeated their experiment with 0.5 µl farnesol on 1st day pupae as well as on 7th day pupae. Previously farnesol had only been tested on 1st day pupae. We got modified adults also when farnesol was injected on the 7th day.

We measured incorporation of uridine-2-^14^ into RNA of pupae which had received 0.5 µl farnesol on their first day of pupation and uridine on the 1st and 7th days. There was no inhibition in uridine incorporation into RNA.

Farnesol injected with radioactive glycine on the 1st and 7th day of pupation, had the same effect as actinomycin D.
It is conceivable that since actinomycin D and farnesol have the same morphological effect, both drugs act on the same biochemical process which is at present unknown to us. The fact that farnesol does not affect overall RNA synthesis indicates that farnesol does not interfere with energy metabolism.

There is a possibility that farnesol affects some specific aspect of RNA metabolism not measurable under our experimental conditions.
SUMMARY

1) 0.16\(\mu\)g actinomycin D caused the formation of modified adults when injected into 1st, 2nd, 3rd, 4th, 5th, 6th and 7th day pupae.

2) The rate of incorporation of uridine-2-C\(^{14}\) into RNA of whole pupae or of slices of dorsal abdominal body wall increased with the day of pupation. 0.16\(\mu\)g actinomycin D inhibited C\(^{14}\) uptake into RNA by 51% on the 1st day and by 67% on the last day of pupation on whole pupae and in dorsal abdominal wall on the last day of pupation the incorporation was inhibited by 75%.

3) In vitro, actinomycin D inhibited uridine-2-C\(^{14}\) incorporation into RNA of dorsal abdominal body wall slices. 1.6\(\mu\)g drug inhibited 80% of the uptake of C\(^{14}\) into RNA, while 0.8 and 0.4\(\mu\)g inhibited 65 and 32% respectively.

4) Thymus DNA had a protective action on the biological effect of actinomycin. Normal adults developed only when DNA was mixed with the drug prior to injection.

5) A mixture of DNA and actinomycin D protected uridine-2-C\(^{14}\) incorporation into RNA in vivo.

6) The presence of DNA in the medium protected uridine-2-C\(^{14}\) incorporation into RNA of dorsal abdominal body wall in vitro.

7) DNA as well as deoxyguanosine reversed the inhibition of uridine-2-C\(^{14}\) incorporation into RNA by actinomycin D.

8) Actinomycin D was without effect on uptake of glycine-1-C\(^{14}\) by protein during the first 6 days of pupation. On the 7th day it
prevented the drop in specific activity of protein observed in the control.

9) Farnesol had the same biological effect as actinomycin D, it also had the same effect on incorporation of glycine into protein. No inhibition could be detected in incorporation of uridine-2-$^{14}$C into RNA.
DISCUSSION

It is well established that during metamorphosis respiration in insects follows a U-shaped curve. We found this type of curve when respiration was measured in dorsal abdominal body wall slices in vitro. Since no measurable aerobic or anaerobic glycolysis could be detected and there was no incorporation of C\textsuperscript{14} from precursors into protein and nucleic acids under anaerobic conditions, we assured that all the energy required for protein and nucleic acid synthesis is derived from respiration. The fact that respiration in the dorsal abdominal body wall followed a U-shaped curve, indicated that at least in this tissue, the typical respiratory curve is not associated with cell division, since no cell division occurs in this tissue during metamorphosis; nor is it associated with changes in cell population or with the formation of new types of cells. The two peaks of the respiratory curve on the first and last days of pupation coincide with the two peaks of the ecdysone titre (307). It is not clear whether ecdysone has a direct effect on respiration in insects. It has been shown that administration of ecdysone into hibernating cecropia pupae initiates adult development, the first sign of which, is a rise in respiration (136). Moreover, there is an increase in Q\textsubscript{02} in cardiac muscle slices incubated with ecdysone. (43).

While the respiration followed a U-shaped curve during metamorphosis, there was a steady rise in the rate of incorporation of C\textsuperscript{14} from precursors into nucleic acids in vivo and in vitro. Uptake of C\textsuperscript{14} into protein in whole pupae and in slices of dorsal abdominal
body wall, however, exhibited a sharp rise in the first day as compared to larvae, the rate of incorporation remaining steady until the emergence of the adult.

Since the pool of free nucleotides which is very small, as well as the pool of free glycine remain stable during metamorphosis (125, 197), the fact that we found an increase in the rate of radioactive glycine incorporation into nucleic acids indicated an increase in the rate of purine synthesis. This is supported by the demonstration that all the radioactivity in the RNA hydrolyzate of tissue incubated with glycine, was in the adenylic and guanylic acid residues. The incorporation of C\(^{14}\) from formate and from glucose into nucleic acids of pupae is further evidence for de novo purine synthesis. Moreover, we were able to show that pupal homogenate reduces TPN and that after the enzymatic reaction, the incubation mixture gave a positive orcinol test.

Although insect tissues incorporate adenine-8-C\(^{14}\), uridine-2-C\(^{14}\) and thymidine-2-C\(^{14}\) into nucleic acids, radioactive glycine is incorporated about 100 times more efficiently, calculated on the basis of \(\mu\)mole atom C\(^{14}\) incorporated. This cannot be explained by failure of the adenine to be taken up by the acid soluble fraction. 90% of the adenine in this fraction is converted into adenine nucleotides (Table 16). More than 80% of the label in the acid soluble fraction was found in ATP, 5 - 10% in ADP and only trace amounts were detected in AMP. The preferential utilization of glycine for nucleic acid synthesis was observed in normal mammalian tissues. This was explained by the breakdown of purine bases. For insects this explanation is not
valid. There is a possibility that when adenine is administered
purine synthesis is inhibited by a feedback mechanism, thereby
inhibiting the formation of guanylic acid. As the pool of free
nucleotides is very small during metamorphosis, under these condi-
tions the amount of available GTP may serve as the limiting factor
in RNA synthesis.

Efficiencies for utilizing available ATP for protein
biosynthesis (measured as glycine-1-Cl\textsubscript{14} uptake by protein) and for
RNA synthesis (measured as uridine-2-C\textsubscript{14} uptake into RNA), were
calculated according to Quastel and Bickis (211). As these authors
pointed out, the efficiency quotient can be equally well expressed
in terms of moles of amino-acid or nucleoside incorporated per mole
ATP. A simple calculation, when the experimental conditions are
known, will give this ratio. However, the ratio used was preferred
for the present, until it is established that the label in protein
is due only to amino-acid incorporated and that in RNA only to
nucleoside incorporated. A common feature for the efficiency
quotients, for RNA and protein biosynthesis in vivo and in vitro
is that their values are low at the beginning of pupation, rising
continuously, reaching a peak on the 5th day of pupation and then
dropping sharply to low levels at the end of pupation (Figs. 14-18).
In whole animals this could be explained by changes in cell population.
Larval cells divide giving rise to pupal cells, which differ in their
efficiency from their ancestors. Additional cell divisions give rise
to adult cells which in turn differ from pupal cells in their efficiency.
The "dilution" of the cell generations could give rise to the typical
efficiency curves observed. This explanation cannot be applied for values obtained with dorsal abdominal body wall slices, since no cell division occurs in this tissue during pupation. Here the changes in the efficiency for utilizing ATP for protein and RNA synthesis are controlled by a mechanism not associated with cell division. Quastel and Bickis (211) have shown that in the development of embryo into adult organs a marked drop takes place in the ability of tissue to incorporate glycine into protein although energy is available. They suggested that one possible cause for the diminished efficiency in adult tissue could be the development in the adult tissue of new enzyme systems dependent upon ATP which are needed for the special function of these tissues. For mammalian tissues this suggestion is supported by discovering that the activity of many enzymes increases during the development of the tissues in which they occur (257). In insects, many enzymes have been shown to have a U-shaped activity curve during pupation. New enzymes such as the enzyme responsible for the formation of eye colour in *Drosophila* and wing colour in lepidopteran insects, develop during metamorphosis. Their activity reaches maximal values at the end of pupation. The holometabolous insect is a multi-form organism in its post-embryonic life, when it changes its form three times, from larva to pupa to adult. These forms are genetically controlled. During the pupal period larval tissues undergo reorganization to adult tissues and organs which take on new functions and forms. Reorganization of tissues in biological systems is accompanied by high levels of efficiency for protein and nucleic acid synthesis. This also follows from the finding of Quastel and Bickis (211), since the vertebrate
embryo is also a multiform organism, the different forms of which are genetically controlled.

Treatment with colchicine has been shown to change the post-embryonic differentiation of insect antennae (259). It was shown (270) by the radioautographic technique that colchicine depressed the number of grains in guinea pig epidermal tissue, which had been incubated with tritiated thymidine. This together with the facts that colchicine changed post embryonic differentiation in insects (250) and has been shown to be a mutagenic agent for coliform bacteria (271) and for the fungus Aspergillus spp (272), that colchicine increased the radio sensitivity in the protozoan Paramecium (272) and caused malformation in the chick embryo (272), suggested that colchicine may act on nucleic acid metabolism. We therefore tested the effect of this drug on the differentiation of pupa into adult and its effect on nucleic acid metabolism. 10\(^7\) colchicine arrests differentiation of pupa into adult insect, the pupa remaining alive for 2-3 weeks. The same concentration of colchicine inhibits DNA synthesis (Figs. 20, 21) (measured as incorporation of radioactive precursors) and stimulated RNA synthesis (Fig. 19, Table 14). The effect of colchicine on nucleic acid metabolism in vivo can first be detected three days after administration of the drug to first day pupae. There is a stimulation of RNA synthesis even with 200\(^7\) colchicine. With this dose of drug the stimulation occurs three days after the injection. 200 colchicine when injected into first day pupae cause an inhibition in RNA synthesis on the 7th day of pupation.

In Tenebrio pupae the biochemical effect of the colchicine in vivo is first detected three days after administration. The toxic
effect of lethal doses of the drug in mammals are first apparent one week after administration. In both cases there is a lag period before the effect of the drug is observed. Therefore, in order to demonstrate the biochemical effect of colchicine in vitro it is necessary to raise the concentration of the drug.

Though colchicine inhibits nucleic acid biosynthesis, the drug does not affect energy metabolism. This may be concluded from the fact that the concentration which inhibits DNA synthesis in vivo is without effect on protein synthesis. Moreover, when dorsal abdominal body wall slices are incubated with adenine-8-C\textsuperscript{14} and 10 mM colchicine, the uptake of label by the acid soluble fraction as well as the amount of ATP formed were the same as in the control (Table 16).

In Tenebrio pupae colchicine primarily inhibits DNA synthesis and to a lesser extent RNA synthesis. The same was found to be the case with mammalian tissues. 1 mM drug had no effect on respiration, inhibiting mainly DNA synthesis and to a slighter extent RNA synthesis. (Measured as adenine-8-C\textsuperscript{14} incorporated, Table 15). Since colchicine inhibits DNA synthesis and cell division without affecting energy metabolism, the question arises why it prevents differentiation in a tissue such as the dorsal abdominal wall, whose cells do not divide during metamorphosis and in which no DNA synthesis occurs during this period. When first day pupae were injected with colchicine (200\mu g) it was found that by the 7th day of pupation a 50% inhibition had occurred in RNA synthesis (incorporation of uridine-2-C\textsuperscript{14}). RNA synthesis was also inhibited when actinomycin D was administered to 7th day pupae (67% Table 22). Simultaneously with the inhibition in
RNA synthesis, development of this tissue was arrested. It is therefore possible that in the dorsal abdominal body wall the inhibition in RNA synthesis is responsible for the arrest in differentiation.

When Ehrlich ascites carcinoma cells were incubated in vitro with colchicine and thymus DNA, the latter provided full protection for adenine-8-C\textsubscript{14} incorporation into RNA and DNA (Table 18). These results suggest that colchicine may inhibit DNA synthesis by combining with the DNA primer (template) in a specific manner, thereby preventing DNA from serving as a primer mainly for DNA synthesis and to a slight degree for RNA synthesis. Such a combination is known to occur with mitomycin C and DNA (308). When DNA-dependent RNA synthesis is inhibited by actinomycin D it is believed that the latter inhibits RNA synthetase by combining with the DNA primer (template). It has been suggested by Reich et al (292) that enzymes catalyzing DNA biosynthesis and DNA dependent RNA biosynthesis, differ significantly in their respective stereochemical relationship to the DNA model.

Evidence for the interaction of colchicine with DNA is provided by the fact that when both substances were mixed, there was a significant shift in the optical rotation observed, from that expected by calculating the sum of the separate rotations of the two substances.

These experimental results strongly suggest that colchicine inhibits DNA synthesis by combining with the DNA primer in a specific manner. However, they do not rule out the possibility that the observed inhibition in the incorporation of adenine-8-C\textsubscript{14} and thymidine-2-C\textsubscript{14} into DNA is not due to the inhibition of deoxycytidilic-, deoxyadenylic-, deoxyguanulic- or deoxythymidilic - kinases. Moreover, our observations
do not eliminate the possibility of inhibition in formation of deoxynucleotides or inhibition of DNA polymerase by direct combination with the enzyme.

Concentrations of colchicine which were used in our in vivo and in vitro experiments were higher than those which are known to arrest mitosis. However, for practical purposes, the range of concentrations similar to the range we used is employed. It was shown (258) that when colchicine is applied to an isolated single grasshopper nerve cell during prophase, metaphase was arrested. Cells at prophase are after the S period of the mitotic cycle and contain twice the amount of DNA present at interphase. No DNA synthesis occurs during this period. The mitotic inhibition, cannot be attributed to inhibition of DNA synthesis. Colchicine is widely used for the estimation of mitotic activity. The drop in DNA synthesis which occurs in cells incubated in the presence of colchicine may be due to an arrest in the S period with an incomplete replication of DNA, or failure of cells newly arrived at this stage to start DNA synthesis. The inhibition in DNA synthesis indicates that the rate of synthesis is slowed so that even if the normal DNA complement were to be reached, the number of cells arriving at metaphase per unit time would be less. It follows that the counting of metaphase figures after colchicine block is very likely to give an underestimate of mitotic activity.

Actinomycin D is an inhibitor of DNA-dependent RNA synthesis. The first species of RNA's to be inhibited are the rapidly-labelled RNAs, the so called messenger RNAs. In bacteria and mammalian tissues the inhibition is due to interaction of the antibiotic with DNA and the
inhibitory effect of the drug can be prevented by the addition of DNA or deoxyguanosine to the incubation medium. The demonstration that in pupae of *Tenebrio* actinomycin D inhibits RNA synthesis (incorporation of uridine-2-C\(^{14}\)) and that RNA synthesis can be protected in vivo by thymus DNA, indicates that in our system the antibiotic acts in the same manner as in mammalian and bacterial systems. When 0.16 μg actinomycin D were injected into first day pupae, modified adults developed; in which the head and thorax are essentially adult, while the abdomen and wings remain pupal like. The same results were obtained when the drug was injected on the remaining days of pupation. 0.16 μg actinomycin D inhibited uridine incorporation into RNA by 51-67% (Fig. 29). The fact that actinomycin D when injected into first day pupae gave rise to the same modified adults as those injected on the last day of pupation suggests that the mRNA for the development of adult head and thorax is present in the animal from the first day of pupation. Revel and Hiatt (303) showed that an inhibition of 19% of total RNA synthesis by actinomycin D (measured as incorporation of C\(^{14}\) orotic acid) is sufficient to inhibit mRNA production in liver nuclei. When chick embryos containing 11-13 somites were cultured for 48 hours in media containing actinomycin D, the antibiotic had no effect on the morphogenesis of the head region, the main disturbance was observed in the posterior part of the body (309).

When thymus DNA was mixed with actinomycin D prior to injecting into pupae, the latter developed into normal adults. This protection did not occur when DNA and actinomycin were injected separately (table 22). However, uridine-2-C\(^{14}\) incorporation into RNA was
protected to some extent even when DNA and drug were injected separately and completely when both substances were injected together. It follows that if RNA synthesis is a prerequisite for normal morphogenesis, a 31% inhibition is sufficient to bring about the formation of modified adults.

The inhibition of RNA synthesis by actinomycin is fully reversible, as can be seen in Fig. 22. DNA as well as deoxyguanosine were able to reverse the effect of actinomycin D. This result is in agreement with the observation of Reich et al (292) who found that DNA-virus preincubated with the antibiotic may become viable when added to cells, presumably due to the competition of the cellular DNA for the viral bound actinomycin D.

The stability of the protein synthesizing units during metamorphosis was examined using actinomycin D. First day pupae were injected with the amount of antibiotic which inhibited uridine-2-C\textsuperscript{14} incorporation into RNA by 51 - 67%. Glycine-1-C\textsuperscript{14} was injected at 24 hour intervals. The specific activity of the protein was the same in the experimental group as in the control during the first six days of pupation (Table 24). On the 7th day of pupation a drop occurred in the specific activity of protein in the control group. This is probably due to the fact that new adult cuticular proteins which contain very little glycine, are being formed on the 7th day. Actinomycin D prevented the drop in specific activity. It has been shown (42) that 46% inhibition of incorporation of labelled uridine into RNA by actinomycin inhibits amino-acid incorporation into protein in a mammalian system when mRNA is not stable. We, therefore, suggest that
during pupation the protein forming units are stable. Messenger RNAs responsible for the development of the posterior part of the body are formed on the last day of pupation. This appears likely since actinomycin D arrests development in this region when injected into 7th day pupae. However, mRNAs for the formation of head and thorax are probably present from the first day of pupation but serve as a template for protein synthesis only on the last day of pupation.

The prothoracic hormone, ecdysone, plays a role in metamorphosis. A rise in titer of this hormone occurs in the prepupae with reaching a peak on the first day of pupation, declining again and rising on the last day of pupation. Administration of the hormone into ligated last instar larvae of Calliphora causes puparium formation (8). When Cecropia pupae undergoing diapause are treated with ecdysone they develop into adults (136). Isolated abdomens also undergo metamorphosis under the influence of ecdysone. These results together with the demonstration by Clever (25,26) that administration of ecdysone causes a definite pattern of localized puffs on the giant chromosomes of the salivary glands of Diptera lead Karlson (8,310) to suggest that ecdysone acts directly on the DNA (gene) inducing the formation of specific mRNAs. Recently, Sekeris and Karlson (137) correlated the induction of DOPA-decarboxylase in prepupae of Calliphora with the rise in ecdysone titer and have shown that metabolic inhibitors acting on RNA synthesis or protein synthesis would delay the induction of the enzyme. However, one has to bear in mind that ribonuclease induces many puffs in salivary gland chromosomes of Drosophila. The induction of these puffs is in definite sequence (311). Puffs may be
induced by other means such as anaerobiosis, temperature shock and uncouplers of oxidative phosphorylation (312). There is no doubt that ecdysone can cause puffs on the giant chromosomes of Diptera but the specificity has to be proved. Our experimental evidence with actinomycin D suggests that mRNAs for the head and thorax exist within the pupae from the first day of pupation and that these messengers are stimulated to serve as templates for protein synthesis on the last day of pupation. If ecdysone is responsible for the transformation from pupa to adult, its influence on the formation of head and thorax is not associated with the induction of new mRNA but with activation of pre-existing messengers. The possibility that ecdysone may have a direct effect on protein synthesis not associated with mRNA production is supported by the demonstration by Burdette (44,313) that cell free protein synthesizing systems from human and murine liver, increase their ability to incorporate leucine-1-C\(^{14}\) by 92% in the presence of ecdysone.

Our suggestion that mRNAs may exist within the cell in a non-active form is supported by the finding of Revel and Hiatt (303) who showed that newly synthesized mRNA remains in the nucleus for as long as 48 hours while the ribosomes are saturated with old messenger. Further evidence that mRNAs may exist in an inactive form within the cell is provided by the experiments of Gross and Cousineau (278) and Gross et al (314). They showed that incorporation of labelled amino acids into proteins of fertilized sea-urchins eggs, continued for seven hours after synthesis of RNA had been stopped by actinomycin. Under these conditions cell division continued but cell differentiation was arrested. The authors claimed that mRNAs for the formation of protein
involved in cell division is present at fertilization and are activated by fertilization. They maintained that only relatively small quantities of mRNAs for "differentiation" protein are synthesized after fertilization. These authors concluded that new mRNAs are required for normal development beyond the early blastula. We assume that the mRNAs for head and thorax development are present in the pupa from the first day of pupation but do not serve as a template until the seventh day. The failure to serve as a template is not likely to be because RNA polymerase or free binding sites on microsomes are limiting factors. Most cell free synthesizing systems respond to addition of mRNA. Ecdysone may play a role in metamorphosis by activating protein synthesis through exposing preexisting mRNAs to serve as a template for RNA polymerase. A similar idea has been put forth by Hamilton (315) to explain the sequences of RNA and Protein synthesis. During early estrogen action Hamilton found that actinomycin D suppresses estrogen-induced synthesis of RNA to levels below those of controls, but protein synthesis in this case is restricted to control values. Since actinomycin D in hormone-free controls limits protein synthesis to lower levels, and since combined estrogen-puromycin-actinomycin D treatments give maximum inhibition of RNA and protein synthesis, evidence exists for a component of uterine hormone response which is not sensitive to actinomycin D, but is sensitive to puromycin.
1) The incorporation of glycine-1-$\text{Cl}^{14}$ into protein and nucleic acids and the $Q_{O_2}$ values in dorsal abdominal slices and minced preparations of first day pupae were similar in Krebs-Ringer and in "Tenebrio Medium". All values were higher in the slices.

2) 100 - 145 mM sodium chloride was the optimal range for uptake of radioactivity into protein and nucleic acids. $Q_{O_2}$ values, oxidation of glycine and the incorporation of label into the ethanol soluble fraction were not affected in the range 0 - 145 mM sodium chloride.

3) $K^+$ concentration ranging from 0 - 100 mM had no effect on glycine uptake into protein nucleic acids and the ethanol soluble fraction. Oxidation of glycine was inhibited in media containing a high concentration of $K^+$.

4) $Ca^{++}$ had no effect on incorporation of glycine into protein, nucleic acids or the ethanol soluble fraction, nor did it alter the oxidation of glycine.

5) 0 - 10 mM $Mg^{++}$ in the medium did not influence the uptake of radioactivity into protein, nucleic acids or the ethanol soluble fraction.

6) Glycine metabolism was not altered in the pH range of 6.2 - 7.8. It was the same in the absence of buffer. Results did not differ in phosphate and bicarbonate buffers.

7) No measurable anaerobic or aerobie glycolysis could be observed.
8) The addition of glucose had no effect on respiration nor on glycine-1-C\textsuperscript{14} incorporation into protein and nucleic acids.

9) Oxygen is essential for glycine uptake by the latter fractions.

10) More glycine is incorporated in first day pupae than in larvae.

11) A U-shaped respiration curve was obtained when whole pupae and when tissue slices were incubated at 28\textdegree C.

12) A rise in the rate of incorporation of glycine-1-C\textsuperscript{14} into protein in the head and thorax was observed in vitro during metamorphosis; while in the abdomen the rate did not change till the last day of pupation and then dropped. The rate of incorporation of glycine was low in larvae and adult thorax but high in adult abdomen.

13) The rate of incorporation of radioactive glycine into protein in the whole pupa is steady in vivo till the last day of pupation and then drops.

14) There was a steady rise in incorporation of glycine-1-C\textsuperscript{14} into nucleic acids during metamorphosis in vitro, which drops in the adult in the head and thorax and remains high in the abdomen.

15) Formate-C\textsuperscript{14} was taken up into the nucleic acid fraction and protein of first day pupae.

16) Radioactive glucose was incorporated into protein and nucleic acids in vitro. At a concentration of 5mM, the uptake of glucose into the ethanol soluble fraction was high, but the extent of its oxidation was negligible.

17) Brain cortex slices oxidize 52 fold more glucose from the intracellular available pool, than does insect tissue.
18) Glucose-6-phosphate dehydrogenase and 6-phosphogluconate activities were demonstrated in a homogenate of thorax from the 7th day pupa. At the end of the incubation the mixture gave a positive orcinol reaction.

19) The rate of glycine incorporation into RNA in vitro was about 100 fold higher than that of adenine. This difference was not due to failure in uptake of adenine. Neither glycine nor adenine were incorporated into DNA of dorsal abdominal wall slices.

20) Radioactive adenine and uridine were incorporated into RNA in vitro at a steady rate during pupation. An increasing rate of incorporation into RNA was observed in vivo during pupation.

21) About 50% less adenine was incorporated into DNA than RNA in dorsal and ventral abdominal walls of pupae in vivo. Thymidine was incorporated into this tissue in vivo.

22) Efficiency quotients for glycine incorporation into protein in vivo and in vitro and for uridine incorporation into RNA in vivo and in vitro were low at the beginning of pupation, rising steadily reaching a peak on the 5th day and dropping to low levels at the end of pupation.

23) Colchicine at concentrations of 10 - 400 μg/pupa arrested differentiation into the adult and prolonged the pupation period from 7 days to 2-3 weeks.

24) 10μg colchicine inhibited adenine-8-C\(^{14}\) incorporation into DNA, stimulating incorporation into RNA, 3 days after administration of the drug to first day pupae.

25) Three days after administration of 10 μg colchicine, inhibition of thymidine-2-C\(^{14}\) incorporation into DNA was observed.
26) 200γ colchicine stimulated uridine-2-C\textsuperscript{14} incorporation into RNA 3 days after administration of drug to first day pupae. At the end of pupation uridine-2-C\textsuperscript{14} incorporation was inhibited.

27) 5 - 10 mM colchicine did not inhibit the O\textsubscript{2} uptake nor incorporation of radioactive adenine by the acid-soluble fraction in dorsal abdominal body wall slices in vitro. ATP formation was also not affected. These concentrations of drug inhibited incorporation of radioactive adenine, uridine and glucose into RNA.

28) The inhibitory effect of colchicine on uptake of radioactive precursors into RNA was more pronounced with uridine and glucose than with adenine.

29) The rate of incorporation of radioactive precursors into RNA in dorsal abdominal body wall in vitro was linear with time up to 2 hours. Colchicine inhibited the velocity of the enzymatic reaction. No lag was observed prior to inhibition. In vivo, the velocity of the enzymatic reaction for incorporation of uridine into RNA was altered after 2 hours incubation in colchicine treated animals and in the control animals.

30) 1 mM colchicine inhibited the incorporation of radioactive adenine in vitro into DNA of mammalian tissues. Incorporation into RNA was only slightly inhibited. Respiration and uptake of radioactivity by the acid soluble fraction were not affected.

31) Thymus DNA protected adenine incorporation into DNA and RNA in Ehrlich ascites carcinoma cells, when the latter were incubated in the presence of colchicine.

32) Measurements of optical activity of DNA, colchicine and a mixture of both compounds indicated that combination of colchicine and DNA had occurred in solution.
33) Sarcomycin at a level of 60 and 120 \( \gamma \)/pupa completely arrested development of 1st day pupae, the latter surviving for 2-3 weeks.

34) 100 \( \gamma \) mescaline per pupa completely arrested differentiation of 1st day pupae. 80 and 160 \( \gamma \) drug inhibited incorporation of adenine-8-C\(^{14} \) into DNA by 53 and 55\% respectively. Incorporation into RNA was not affected.

35) 0.16 \( \gamma \) actinomycin D caused the formation of modified adults when injected into 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th day pupae.

36) The rate of incorporation of uridine-2-C\(^{14} \) into RNA of whole pupae or of slices of dorsal abdominal body wall increased with the day of pupation. 0.16 \( \gamma \) actinomycin D inhibited C\(^{14} \) uptake into RNA by 51\% on the 1st day and by 67\% on the last day of pupation on whole pupae and in dorsal abdominal wall on the last day of pupation the incorporation was inhibited by 75\%.

37) In vitro, actinomycin D inhibited uridine-2-C\(^{14} \) incorporation into RNA of dorsal abdominal body wall slices. 1.6 \( \gamma \) drug inhibited 80\% of the uptake of C\(^{14} \) into RNA, while 0.8 and 0.4 \( \gamma \) inhibited 65 and 32\% respectively.

38) Thymus DNA had a protective action on the biological effect of actinomycin. Normal adults developed only when DNA was mixed with the drug prior to injection.

39) A mixture of DNA and actinomycin D protected uridine-2-C\(^{14} \) incorporation into RNA in vivo.

40) The presence of DNA in the medium protected uridine-2-C\(^{14} \) incorporation into RNA of dorsal abdominal body wall in vitro.

41) DNA as well as deoxyguanosine reversed the inhibition of uridine-2-C\(^{14} \) incorporation into RNA by actinomycin D.
42) Actinomycin D was without effect on uptake of glycine-1-\textsuperscript{14}C by protein during the first 6 days of pupation. On the 7th day it prevented the drop in specific activity of protein observed in the control.

43) Farnesol had the same biological effect as actinomycin D, it also had the same effect on incorporation of glycine into protein. No inhibition could be detected in incorporation of uridine-2-\textsuperscript{14}C into RNA.
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1) A U-shaped respiration curve for insect tissue in vitro was first shown.

2) Under anaerobic conditions no incorporation of radioisotopes into protein or nucleic acids could be detected during metamorphosis.

3) Glycine-$^{14}$C incorporation into the total nucleic acid fraction and into RNA, glucose-$^{14}$C incorporation into RNA, Adenine-8-$^{14}$C incorporation into RNA and DNA, and thymidine-$^{14}$C incorporation into DNA were demonstrated for the first time with insect tissues.

4) It was shown that the rate of uptake of radioisotopes in vivo by RNA and DNA increases as metamorphosis proceeds and drops at the end of pupation. In vitro radioactive precursors were incorporated into RNA at a steady rate during pupation.

5) The rate of glycine incorporation into RNA in vitro was 100 fold higher than that of adenine. This was not due to failure in uptake of adenine into the acid soluble fraction nor to the failure of converting adenine to ATP.

6) Efficiency quotients for glycine incorporation into protein in vivo and in vitro and for uridine incorporation into RNA in vivo and in vitro were demonstrated for the first time in an insect system. The efficiency for utilizing ATP for protein and RNA biosynthesis in all cases examined are low at the beginning of pupation, rising steadily to a peak on the 5th day of pupation and dropping to a low level at the end of pupation.

7) Colchicine inhibits the differentiation of T. molitor pupae into adults and prolongs the pupation period to 2 - 3 weeks.
8) In pupae colchicine inhibited adenine-8-C\textsubscript{14} incorporation mainly into DNA and after prolonged exposure to the drug RNA synthesis was also inhibited. The inhibition of DNA synthesis (incorporation of radioisotopes) was shown also with mammalian tissues \textit{in vitro}.

9) Thymus DNA protected incorporation of adenine-8-C\textsubscript{14} into RNA in the presence of colchicine.

10) Measurements of optical activity of DNA, colchicine and mixture of both compounds indicated that combination of colchicine and DNA had occurred in solution.

11) Mescaline inhibited the incorporation of adenine-8-C\textsubscript{14} into DNA but not into RNA \textit{in vivo}.

12) Injection of actinomycin D into pupae caused the formation of modified adults. Actinomycin was effective on all days of pupation.

13) Actinomycin D inhibited uridine-2-C\textsubscript{14} incorporation into RNA in pupae \textit{in vivo} and \textit{in vitro}.

14) Thymus DNA when mixed with actinomycin D prior to injecting into pupae, protected the latter from the inhibitory effect of the drug on morphogenesis. Normal adults developed.

15) The incorporation of uridine into RNA in the presence of actinomycin can be completely protected by mixing the antibiotic with thymus DNA prior to injecting into pupae. When DNA and actinomycin D were injected separately into pupae only partial protection of uridine incorporation into RNA occurred.
16) In vitro, the presence of thymus DNA in the medium protected uridine incorporation into RNA in dorsal abdominal body wall slices, from the inhibitory effect of actinomycin D. DNA and deoxyguanosine are able to reverse the inhibition in the uptake of uridine.

17) Concentrations of actinomycin D which inhibited uridine incorporation into RNA by 50 - 67% had no effect on the incorporation of glycine into protein for the first six days of pupation. On the 7th day, however, the specific activity of the protein was affected by the drug. This occurred when the drug was injected on the first and on the seventh day of pupation.