

Effects of Actinomycin D on Nucleic Acid Metabolism and Protein Biosynthesis during Metamorphosis of *Tenebrio molitor* L.

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1. Injection of 0.16 μ g. of actinomycin D into pupae of the beetle *Tenebrio molitor* L. results in the development of modified adults in which the head and thorax are essentially adult while the abdomen and wings remain pupal-like. It is suggested that the messenger RNA for the development of head and thorax is present in the animal from the first day of pupation. 2. Injection of 0.16 μ g. of actinomycin D brings about 51–67% inhibition of labelled uridine incorporation into RNA. 3. When thymus DNA is mixed with actinomycin D before injection into pupae the latter develop into normal adults. This protection does not occur when DNA and actinomycin D are injected separately. 4. The inhibition of incorporation of labelled uridine into RNA by actinomycin is diminished to some extent when DNA and actinomycin D are injected separately and abolished if they are injected together. 5. Inhibition of RNA synthesis by actinomycin D *in vitro* is fully reversible. DNA or deoxyguanosine can reverse the effect of actinomycin D. 6. Incorporation of labelled glycine into protein is not affected by actinomycin D injection during the first 6 days of pupation. On the seventh day it becomes diminished in control pupae but this effect is prevented by actinomycin D. It is suggested that the template for protein synthesis is stable during the first 6 days of metamorphosis and that on the seventh day there is a qualitative change in the protein synthesized on the template.

It was noted in 1958 that actinomycin D inhibits RNA, but not DNA, synthesis in *Bacillus subtilis* (Slotnick, 1958), and interferes with RNA synthesis in HeLa cells (Milton, Goldstein, Slotnick, Hillman & Gallagher, 1959).

Reich, Franklin, Shatkin & Tatum (1961) showed that actinomycin D inhibits the incorporation of tritiated cytidine into RNA, but does not interfere with the incorporation of tritiated thymidine into DNA of L cells in tissue culture. They further showed in strains L-929 and L-2 mouse fibroblasts that actinomycin D will suppress the biosynthesis of normal cellular RNA and not the biosynthesis of DNA and protein for prolonged periods. A binding takes place between actinomycin D and template DNA (Reich, Franklin, Shatkin & Tatum, 1962; Bickis & Quastel, 1962), accounting for the suppression of RNA synthesis. The binding of actinomycin D to DNA is not irreversible since a DNA virus preincubated with

actinomycin D may become viable when added to cells, presumably owing to the competition of the cellular DNA for the viral-bound actinomycin D (Reich *et al.* 1962). In cell-free systems, with bacterial enzymes (Hurwitz, Furth, Malamy & Alexander, 1962) and mammalian enzymes (Goldberg & Rabinowitz, 1962) capable of catalysing the DNA-dependent synthesis of RNA, actinomycin D inhibits the synthesis of polyribonucleotides.

Kersten (1961) showed that high concentrations of deoxyguanosine, and to a smaller extent guanine and adenine compounds, will alter the spectral properties of actinomycin D solutions. This has been confirmed (Wheeler & Bennett, 1962). Bickis & Quastel (1962) have shown that DNA is also active in protecting against the inhibitory effect of the antibiotic on adenine incorporation into RNA in Ehrlich ascites-carcinoma cells. Goldberg, Rabinowitz & Reich (1962) have found, in DNA-dependent RNA synthesis, that the sensitivity of the process to the antibiotic is a function of the guanine and cytidine content of DNA primer. DNA primers, not containing deoxyguanosine

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residues, do not bind actinomycin D, nor is their priming of RNA synthesis inhibited by the antibiotic.

In insects, actinomycin D has been used to investigate the activities of the giant chromosomes of the salivary glands of *Drosophila* in bringing about RNA synthesis. The activities are suppressed by actinomycin D (Reich, 1963). The antibiotic was also used to demonstrate the induction of 3,4-dihydroxyphenylalanine decarboxylase in prepupae of *Calliphora* (Sekeris & Karlson, 1964) and for the investigation of protein synthesis during the diapausing of *Cecropia* (Berry, Krishnakumaran & Schneiderman, 1964).

We have carried out experiments to investigate the effects of actinomycin D on nucleic acid metabolism, protein biosynthesis and morphological changes of *Tenebrio molitor* L. during metamorphosis. During the pupal stage considerable cell differentiation occurs but as there is no intake of food during this period the amino acids and nucleotides needed for these changes have to be provided by the free pool, by new synthesis or by breakdown of existing tissue. Attention has therefore been concentrated on this stage, which thus represents a closed system (except for gas exchange).

MATERIAL AND METHODS

T. molitor was maintained at room temperature on a diet of wheat-bran and oat-flakes (2:1, w/w) (Patterson, 1957). Prepupae were collected from the culture and kept in separate containers. The pupae that emerged daily between 5 p.m. and 10 a.m. were considered as first-day pupae. These were incubated in a highly humid atmosphere at 28°. Under these conditions pupation lasted 7 days, the adults emerging on the eighth day.

Pupae weighing 110–120 mg. were used. For experiments *in vitro* the head and thorax were removed. Dorsal abdominal slices were obtained by means of a Stadie-Riggs tissue slicer. Three such slices were used per incubation flask. Pupae were injected by means of Hamilton microsyringes. Routine injections were made into the blood system through the abdomen. Preliminary experiments have shown that the site of injection is immaterial since injections of actinomycin D into the head, thorax or abdomen brought about the same morphological changes.

Incubations were carried out in Krebs-Ringer phosphate media (Ilan & Quastel, 1966) at 37° in the conventional Warburg manometric apparatus. (Radioactive materials were tipped into the main compartment of the manometric flask from the side tube at zero time.) The reaction was stopped by placing the vessels in crushed ice. After incubation at 37° the tissues were removed and washed in 10 ml. of ice-cold Krebs-Ringer phosphate medium. The tissues were homogenized in 5 ml. of ice-cold 6% (w/v) trichloroacetic acid. After centrifugation the supernatant was extracted three times with 3 vol. of ether and the remaining aqueous fraction was termed the acid-soluble fraction. The acid-insoluble residue was then washed twice with

5 ml. of ice-cold 6% trichloroacetic acid, once with 5 ml. of 95% (v/v) ethanol, once with ethanol-ether (3:1, v/v) and once with ether. RNA was separated from DNA by the Schmidt & Thannhauser (1945) procedure. DNA was precipitated by ice-cold HClO₄ and washed twice with 5 ml. of ice-cold 6% trichloroacetic acid to remove KClO₄. Samples of the acid-soluble fraction and the RNA and DNA fractions were plated on aluminium planchets and counted in a Tracerlab scaler with a Geiger-Müller mica end-window probe (20% efficiency). No correction for self-absorption was necessary.

Protein from dorsal abdominal slices was prepared as described by Quastel & Bickis (1959) with a modification as it was necessary to dispose of the exoskeleton before determining the specific activity of the protein. The tissue was homogenized in 5 ml. of ice-cold 6% trichloroacetic acid and washed with 5 ml. of ether. The protein was dissolved by the addition of 2 ml. of 0.5 N-KOH and incubation of the material for half an hour at room temperature. The exoskeleton was separated by centrifugation. Next 0.2 ml. of 70% (w/v) HClO₄ was added to precipitate the protein, which was then washed twice with 5 ml. of 6% trichloroacetic acid. The specific activity of the protein was then determined by the procedure quoted above (Quastel & Bickis, 1959). A further modification was required when whole insects were used as these have a high lipid content (20–30%). These were homogenized in 10 ml. of 80% (v/v) 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, v/v) at 60° for 1 hr. It was then washed twice with ethanol-ether (3:1, v/v) and once with acetone. To the precipitate was added 2 ml. of 0.5 N-KOH and the mixture was kept for half an hour at room temperature. The exoskeleton was removed and the protein precipitated as described above. The nucleic acids were removed by the method of Schneider (1945). The protein was suspended in acetone and plated on weighed aluminium planchets. The weight of the protein and its radioactivity were determined and corrections were made for self-absorption. Results are expressed in terms of specific activity (counts/min./mg. of protein).

[8-¹⁴C]Adenine sulphate hemihydrate and [1-¹⁴C]glycine were purchased from The Radiochemical Centre, Amersham, Bucks. [2-¹⁴C]Uridine was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. Actinomycin D was a gift from the Merck Laboratories, Montreal, Canada. Thymus DNA that had been prepared by the method of Kay, Simmons & Dounce (1952) was a gift from Dr S. C. Sung of our Laboratory.

The results quoted in the Tables below are the means of at least six separate determinations and the standard deviations from the means are quoted.

RESULTS

Effects of actinomycin D on the differentiation of pupa into adult. Groups of first-day pupae were injected with 1 μ l. containing 0.04 μ g., 0.08 μ g. or 0.16 μ g. of actinomycin D dissolved in sterile 0.9% sodium chloride/pupa. The control group, which had received injections of sterile 0.9% sodium chloride, and the group that had received 0.04 μ g. of actinomycin D/pupa matured into normal adults

after incubation for 7 days at 28°. In the group that had received 0.08 μg . of actinomycin D/pupa only 30% of the insects developed into normal adults. Injection of 0.16 μg . of actinomycin D/pupa modified adult development in all samples injected (40 animals in each group). Photographs of normal and modified *Tenebrio* beetles are given in Fig. 1. 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 3 weeks. They exhibited normal movement but they did not lay eggs.

When 1 μl . containing 0.16 μg . of actinomycin D was injected into first-, second-, third-, fourth-, fifth-, sixth- or seventh-day pupae (24 pupae per group), all pupae developed into modified adults, whereas control pupae developed into normal adults. These results indicated that actinomycin D when injected into first-day pupae does not affect the normal development of the head and thorax. Moreover, injection of actinomycin D on the seventh day of pupation (last day) was sufficient to arrest development of the wings and abdomen.

Effects of actinomycin D on [2-¹⁴C]uridine incorporation into RNA in vivo and in vitro. First-, third-, fifth- and seventh-day pupae were injected

with 0.16 μg . of actinomycin D and [2-¹⁴C]uridine. After incubation for 24 hr. at 28° (Fig. 2) the rate of uridine incorporation *in vivo* into RNA from whole pupae increases with the day of pupation. Injection of 0.16 μg . of actinomycin D inhibits incorporation of uridine into RNA as from the first day by 51% and the inhibition rises during pupation to 67% on the seventh day.

In dorsal abdominal body wall (Fig. 3), 0.16 μg . of actinomycin D inhibits incorporation of uridine into RNA *in vivo* by 51% on the first day rising to 75% on the seventh day.

When dorsal abdominal body-wall slices of first-day pupae are incubated *in vitro* with [2-¹⁴C]-uridine and 1.6 μg . of actinomycin D/ml., incorporation of radioactivity into RNA is inhibited by 80%. In the presence of 0.80 μg . and 0.40 μg . of actinomycin D/ml., uptake of [2-¹⁴C]uridine is inhibited by 65 and 32% respectively (Fig. 4). The percentage inhibitions were determined with the latter concentrations in third-, fifth- and seventh-day pupae and were found to increase with the day of pupation (Table 1).

Effect of thymus DNA on the inhibitory action of

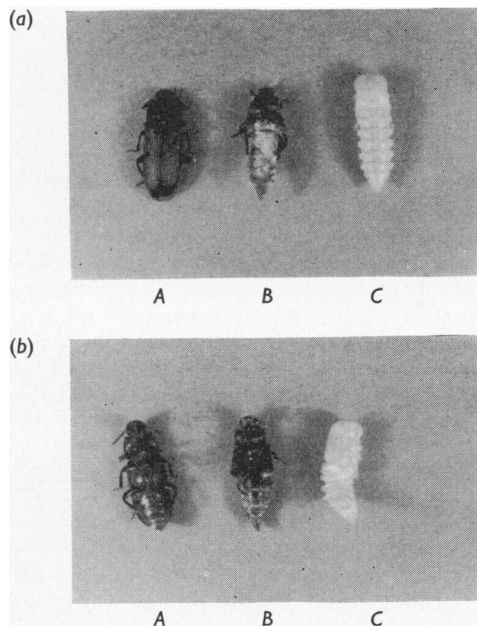


Fig. 1. (a) Dorsal view and (b) ventral view of: A, normal adult; B, modified adult (after injection of 0.16 μg . of actinomycin D); C, normal pupa.

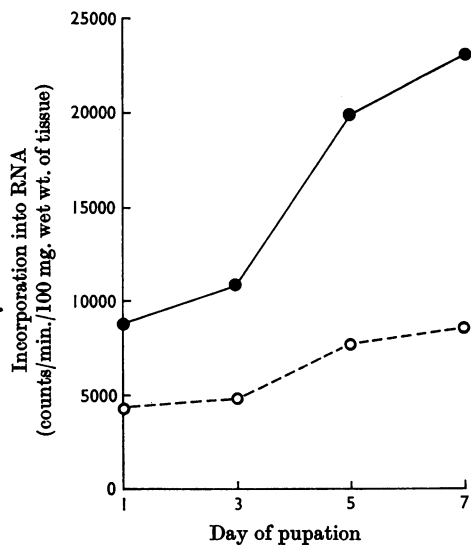


Fig. 2. Incorporation of [2-¹⁴C]uridine into RNA of the whole body during pupation *in vivo* and the effect of actinomycin D. A 0.16 μg . dose of actinomycin D (1 μl .) and [2-¹⁴C]uridine (specific activity 0.2 $\mu\text{C}/12.6 \text{ m}\mu\text{moles}$) (88000 counts/min., 2 μl .) were injected into first-, third-, fifth- and seventh-day pupae. Control insects received 1 μl . of 0.9% NaCl instead of actinomycin solution. The pupae were incubated for 24 hr. at 28° before determination of incorporation of radioactivity into RNA. Results are means of six separate determinations, the standard deviation being $\pm 4\%$. ●, [2-¹⁴C]uridine; ○, [2-¹⁴C]uridine + actinomycin D (0.16 μg .).

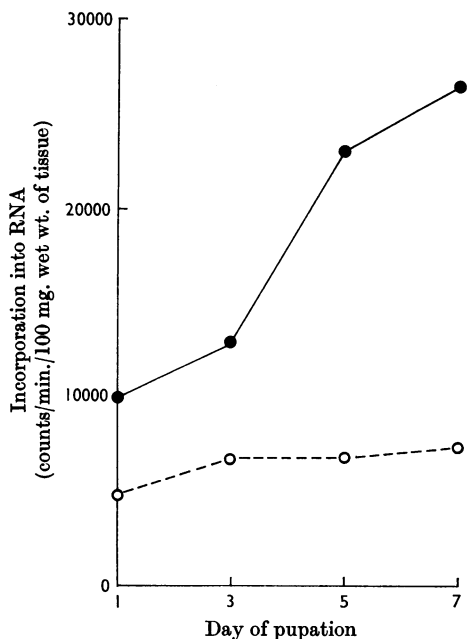


Fig. 3. Incorporation of [2-¹⁴C]uridine into RNA of dorsal abdominal body wall during pupation *in vivo* and the effect of actinomycin D. Experimental conditions were as given in Fig. 2. ●, [2-¹⁴C]uridine; ○, [2-¹⁴C]uridine + actinomycin D (0.16 μg.).

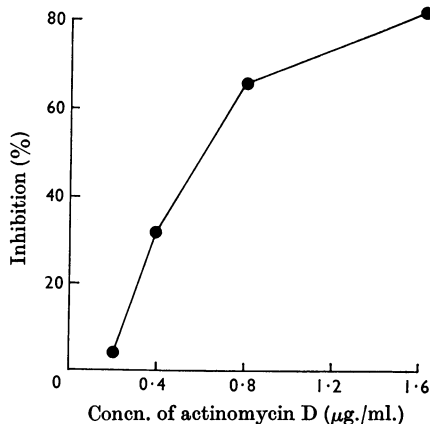


Fig. 4. Effects of concentration of actinomycin D on incorporation of [2-¹⁴C]uridine into RNA of first-day pupae *in vitro*. Dorsal abdominal body wall of first-day pupae was incubated at 37° for 1 hr. in Krebs-Ringer phosphate medium, pH 7.4, in the presence of 10 mM-glucose, actinomycin D and [2-¹⁴C]uridine (405 000 counts/min./vessel, 6.53 μg.). The total volume was 1 ml. The gas phase was O₂. Percentage inhibition was calculated by taking the control value as 2240 counts/min./100 mg. wet wt. of tissue. Results are means of six separate determinations. The standard deviations are similar to those given in Table 1.

actinomycin D. The effect of DNA in preventing the actinomycin D inhibition of incorporation of radioactivity into RNA has been demonstrated *in vitro* with mammalian tissue slices (Bickis & Quastel, 1962), in extracts of HeLa cells (Goldberg & Rabinowitz, 1962) and *in vivo* with bacterial systems (Wheeler & Bennett, 1962). This protective effect of DNA has not, however, been shown in animal tissues *in vivo*.

The protective action of thymus DNA on the biological effect of actinomycin D as well as on the inhibition of [2-¹⁴C]uridine incorporation into RNA *in vivo* is demonstrated by the results presented in Table 2. When 0.08 μg. or 0.16 μg. of actinomycin D is injected into sixth-day pupae the latter develop into modified adults. However, when this amount of actinomycin D is injected together with 80 μg. of DNA, normal adults develop. The same is true when 40 μg. of DNA is injected together with 0.08 μg. of actinomycin D. When DNA is injected first and the actinomycin D administered half an hour later, or vice versa, DNA fails to abolish the inhibitory effect of actinomycin and modified adults are formed. When DNA is injected alone, normal adults emerge. Protection of the pupae from the inhibitory effect of actinomycin D occurs only when DNA and actinomycin D are injected together.

The presence of DNA was also found to protect incorporation of [2-¹⁴C]uridine into RNA *in vivo* when 40 μg. of DNA and 0.08 μg. of actinomycin D are both injected (Table 2). When 0.08 μg. of actinomycin D is injected alone, incorporation of uridine is inhibited by 63% but no inhibition occurs when actinomycin D and DNA are mixed before injection. However, some protection of the incorporation of [2-¹⁴C]uridine into RNA occurs also when DNA and actinomycin D are given separately: a 38% inhibition occurs when DNA is injected half an hour before the actinomycin is injected and 41% inhibition occurs when the order of injection is reversed (Table 2). If the inhibition of RNA synthesis, measured by [2-¹⁴C]uridine incorporation into RNA, is responsible for the modified morphogenesis, then 38% inhibition of the rate of incorporation is sufficient to bring about the formation of a modified adult.

Thymus DNA was found to have a protective effect on [2-¹⁴C]uridine incorporation into RNA when dorsal abdominal body-wall slices were incubated *in vitro* in the presence of 200 μg. and 400 μg. of DNA/ml. together with 0.8 μg. of actinomycin D/ml. (Table 3). When no DNA was present, 0.8 μg. of actinomycin D/ml. inhibited uptake of radioactivity by 70%. The inhibition was 36% in the presence of 200 μg. of DNA/ml., whereas 400 μg. of DNA/ml. gave full protection from inhibition by the actinomycin D.

Table 1. *Effects of actinomycin D in vitro on [2-¹⁴C]uridine incorporation into RNA of dorsal abdominal body wall during pupation*

Tissue was incubated for 1 hr. at 37° in Krebs-Ringer phosphate medium, pH 7.4, in the presence of 10 mM-glucose and 6.5 µg. of [2-¹⁴C]uridine (357 000 counts/min.)/vessel. The gas phase was O₂. The results are the means ± s.d. of six independent determinations.

Concn. of actinomycin D (µg./ml.)	Incorporation into RNA (counts/min./100 mg. wet wt. of tissue)				
	Day of pupation.....	1	3	5	7
0.0		2340 ± 142	2520 ± 154	2610 ± 145	3000 ± 160
0.4		1360 ± 135	1350 ± 147	1165 ± 122	1160 ± 130
0.8		820 ± 74	800 ± 82	765 ± 69	780 ± 67

Table 2. *Protective action of thymus DNA on inhibition of morphogenesis, and incorporation of [2-¹⁴C]uridine into RNA, by actinomycin D*

The total volume injected was 10 µl. Sixth-day pupae were used. [2-¹⁴C]Uridine (specific activity 123 µc/mg.) (88 000 counts/min./pupa) was injected. RNA was determined after incubation for 24 hr. at 28°. Morphological observations were made 48 hr. after injections. Results are means ± s.d. of six separate determinations.

Substances injected	Morphological change	Incorporation into RNA (counts/min./100 mg. wet wt. of tissue)
Actinomycin D (0.16 µg.)	Modified adult	
Actinomycin D (0.08 µg.); 30 min. later DNA (80 µg.)	Modified adult	
Actinomycin D (0.16 µg.); 30 min. later DNA (80 µg.)	Modified adult	
DNA (80 µg.); 30 min. later actinomycin D (0.08 µg.)	Modified adult	
DNA (80 µg.); 30 min. later actinomycin D (0.16 µg.)	Modified adult	
DNA (80 µg.) + actinomycin D (0.08 µg.)	Normal	
DNA (80 µg.) + actinomycin D (0.16 µg.)	Normal	
DNA (80 µg.)	Normal	
0.9% NaCl control	Normal	15 450 ± 450
DNA (40 µg.)	Normal	15 630 ± 480
DNA (40 µg.) + actinomycin D (0.08 µg.)	Normal	14 000 ± 420
DNA (40 µg.); 30 min. later actinomycin D (0.08 µg.)	Modified adult	9 550 ± 375
Actinomycin D (0.08 µg.); 30 min. later DNA (40 µg.)	Modified adult	9 088 ± 405
Actinomycin D (0.08 µg.)	Modified adult	5 896 ± 277

Table 3. *Protective action of thymus DNA on the inhibitory effect of actinomycin D on the incorporation of [2-¹⁴C]uridine into RNA of dorsal abdominal body wall in vitro*

First-day pupae were used. Experimental conditions were as given in Table 1. The results are means ± s.d. of six separate determinations.

Additions	Incorporation into RNA (counts/min./100 mg. wet wt. of tissue)
Control (no addition)	2500 ± 159
Actinomycin D (0.8 µg./ml.)	880 ± 85
DNA (200 µg./ml.) + actinomycin D (0.8 µg./ml.)	1600 ± 132
DNA (400 µg./ml.) + actinomycin D (0.8 µg./ml.)	2400 ± 140
DNA (400 µg./ml.)	2450 ± 152

The following experiment, whose results are given in Fig. 5, was carried out in an attempt to reverse the inhibitory action of actinomycin D on the incorporation of [2-¹⁴C]uridine into RNA *in vitro*. Dorsal abdominal body-wall slices were incubated for 45 min. and then 0.8 µg. of actinomycin D (in 0.1 ml. of 0.9% sodium chloride) was added. Sterile 0.9% sodium chloride alone was added in the control experiment. Then 45 min. later 400 µg. of DNA or 400 µg. of deoxyguanosine (in 0.1 ml. of 0.9% sodium chloride) was added. Samples were then taken every 45 min. for determination of radioactivity in RNA. It was found that both DNA and deoxyguanosine reverse the inhibition by actinomycin D *in vitro*.

Effects of actinomycin D on protein synthesis during pupation. The effects of actinomycin D on [1-¹⁴C]glycine incorporation into protein during

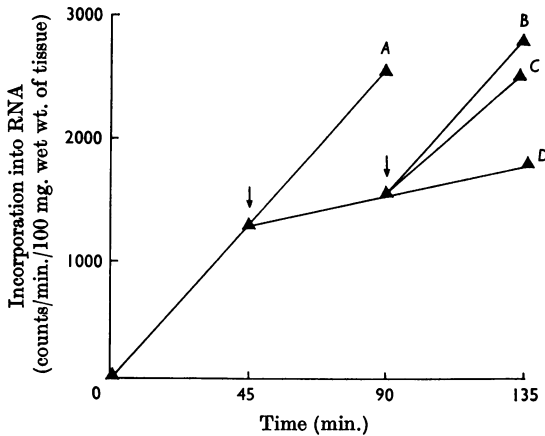


Fig. 5. Reversal of the inhibitory action of actinomycin D on $[2-^{14}\text{C}]$ uridine incorporation into RNA by DNA and deoxyguanosine *in vitro*. Dorsal abdominal body-wall slices of first-day pupae were incubated in Krebs-Ringer phosphate medium, pH 7.4, at 37° for 135 min. in the presence of 10 mM-glucose and $[2-^{14}\text{C}]$ uridine (200 000 counts/min., $3.2\ \mu\text{g.}$). The initial volume was 0.9 ml. The gas phase was O_2 . At 45 min. $0.8\ \mu\text{g.}$ of actinomycin D (in 0.1 ml. of 0.9% NaCl) or 0.9% NaCl alone was added. At 90 min. $400\ \mu\text{g.}$ of deoxyguanosine or $400\ \mu\text{g.}$ of DNA or 0.9% NaCl (0.1 ml.) was added. Determinations were made every 45 min. Results are means of six separate determinations. Standard deviations are similar to those given in Table 3. A, 0.9% NaCl; B, actinomycin D + deoxyguanosine; C, actinomycin D + DNA; D, actinomycin D + 0.9% NaCl.

pupation were investigated. Actinomycin D ($0.16\ \mu\text{g.}$) was injected into first-day pupae, which were then incubated at 28° . Groups were removed from the incubator at 24 hr. intervals, injected with $[1-^{14}\text{C}]$ glycine and replaced in the incubator. Incorporation of glycine into protein was determined 24 hr. after the injection of glycine. The specific activities of the protein (Table 4) were found to be the same in the experimental groups as those in the control groups for the first 6 days of pupation. In the control group, injected with $[1-^{14}\text{C}]$ glycine on the seventh day, the specific activity of the protein was half that observed when injections were made on previous days. In this group, during the 24 hr. of incubation, the pupae had become adults. The specific activity of protein in the group injected with actinomycin D did not show this fall. These pupae developed into modified adults.

DISCUSSION

The present demonstration that, in pupae of *Tenebrio*, actinomycin D inhibits RNA synthesis (incorporation of $[2-^{14}\text{C}]$ uridine) and that RNA

Table 4. Effects of actinomycin D on $[1-^{14}\text{C}]$ glycine incorporation into protein of whole pupae *in vivo*

First-day pupae were injected with $0.16\ \mu\text{g.}$ of actinomycin D in a volume of 1 $\mu\text{l.}$ Control pupae were injected with 0.9% NaCl. Pupae from each day of pupation were injected with $2.5\ \mu\text{l.}$ of $[1-^{14}\text{C}]$ glycine (41 000 counts/min., $2.2\ \mu\text{g./pupa}$). After 24 hr. of incubation at 28° the specific activity of the protein was determined. The results are the means \pm s.d. of at least six separate determinations.

Day of pupation	Specific activity of protein (counts/min./ $\mu\text{g.}$)	
	Control	With actinomycin D injected
1	580 ± 52	575 ± 49
2	595 ± 65	600 ± 55
3	620 ± 45	610 ± 52
4	600 ± 48	615 ± 45
5	590 ± 44	595 ± 48
6	610 ± 47	615 ± 47
7	300 ± 17	610 ± 51

synthesis can be protected *in vivo* by thymus DNA, indicates that in this system the antibiotic acts in a manner similar to that in mammalian and bacterial systems. When $0.16\ \mu\text{g.}$ of actinomycin D is injected into first-day pupae, modified adults develop, in which the head and thorax are essentially adult while the abdomen and wings remain pupal-like. The same results are obtained when the antibiotic is injected on any of the remaining days of pupation. Injection of $0.16\ \mu\text{g.}$ of actinomycin D inhibits uridine incorporation into RNA by 51–67% (Fig. 2). The fact that actinomycin D when injected into first-day pupae gives rise to the same modified adults as those injected on the last day of pupation suggests that the m-RNA* for the development of adult head and thorax is present in the animal from the first day of pupation. Revel & Hiatt (1964) have shown that an inhibition of 19% of total RNA synthesis by actinomycin D (measured by incorporation of $[^{14}\text{C}]$ orotic acid) is sufficient to inhibit m-RNA production in liver nuclei. When chick embryos containing 11–13 somites are cultured for 48 hr. in media containing actinomycin D, the antibiotic has no effect on the morphogenesis of the head region, the main disturbance being observed in the posterior part of the body (Klein & Pierro, 1963).

When thymus DNA is mixed with actinomycin D before injection into pupae, the latter develop into normal adults. This protection does not occur when DNA and actinomycin D are injected separately (Table 2). However, the inhibition by actinomycin D of $[2-^{14}\text{C}]$ uridine incorporation into RNA is diminished to some extent when DNA and

* Abbreviation: m-RNA, messenger RNA.

the antibiotic are injected separately, and abolished completely when both substances are injected together. It follows that, if RNA synthesis is a prerequisite for normal morphogenesis, a 31% inhibition (Table 2) is sufficient to bring about the formation of modified adults.

The inhibition of RNA synthesis by actinomycin D *in vitro* is fully reversible, as shown by the results given in Fig. 5. DNA, as well as deoxyguanosine, can reverse the effect of actinomycin D. This result is in agreement with the observation of Reich *et al.* (1962), who found that DNA virus preincubated with the antibiotic may become viable when added to cells.

The stability of the protein-synthesizing system during metamorphosis was examined by using actinomycin D and [^{14}C]glycine. The rate of incorporation of the latter falls on the seventh day. This fall can be prevented by actinomycin D. It is possible that during pupation the protein synthesized has the same glycine content, per unit weight, until the seventh day, when new types of protein are synthesized with a lower average glycine content. These may be associated with the formation of the new cuticle and the formation of wings. In modified adults this formation does not take place.

These results suggest that the template for protein synthesis is stable during the first 6 days of metamorphosis and that on the seventh day there is a qualitative change in the protein synthesized on the template. The latter change is apparently sensitive to actinomycin D, and under the influence of this substance the initial pupal protein-synthesizing system remains unchanged on the seventh day. It is possible that the m-RNA molecules formed during pupation are stable for the first 6 days of pupation and that on the seventh day a new m-RNA is formed. Presumably the formation of the new m-RNA is blocked by actinomycin D and the initial template is unchanged.

It seems possible that certain m-RNA molecules associated with the formation of head and thorax may exist within the cell without taking part in protein synthesis. This suggestion is supported by the finding of Revel & Hiatt (1964), who showed that newly synthesized m-RNA remains in the nucleus for as long as 48 hr. while the ribosomes are saturated with old m-RNA. Further evidence that m-RNA molecules may exist within the cell that do not take part in protein synthesis is provided by the experiments of Gross, Malkin & Moyer (1964) and Gross & Cousineau (1963). They showed that incorporation of labelled amino acids into proteins of fertilized sea-urchin eggs continues for 7 hr. after synthesis of RNA has been suppressed by actinomycin D. Under these conditions cell division continues but cell differentiation is arrested.

The authors suggest that m-RNA molecules for the formation of protein involved in cell division are present at fertilization and are activated by this process. It is relevant here that Allfrey & Mirsky (1963) have shown that in a mammalian system, containing short-lived m-RNA, actinomycin D at a concentration bringing about 46% inhibition of incorporation of labelled uridine into RNA blocks amino acid incorporation into proteins. In the insect system investigated in the present work, the presence of actinomycin D does not prevent the initial pupal protein-synthesizing system from proceeding. It seems possible that the m-RNA molecules for head and thorax development in *T. molitor* are present in the pupa from the first day of pupation but do not serve as a template until the seventh day.

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