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Title: EFFECTS OF X-IRRADIATION ON DNA SYNTHESIS IN SALIVARY GLAND CELLS OF DROSOPHILA HYDEI

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The effect of X-irradiation on DNA synthesis in larval salivary glands of Drosophila hydei was studied using the technique of liquid scintillation spectrometry and autoradiography. DNA synthesis in salivary glands of synchronized larvae was measured at successive stages of third instar development by in vitro uptake of ^{3}H-thymidine for a period of 30 minutes in ten pooled glands. A "Major Period of DNA Synthesis," hereafter referred to as the "MPS," which lasts for about 5% of larval life, was found in glands just before the mid-point of third instar development. DNA synthesis before and after this period is relatively low.

In vivo exposure of salivary glands before the MPS to acute X-ray doses of 1250, 2500, 5000 and 10,000 R resulted in stimulation of DNA synthesis shortly after irradiation, as determined by liquid scintillation counting. Autoradiographs prepared from glands exposed to 1250 R of X-rays before the MPS show an increase in the number of

cells in very active DNA synthesis at the anterior part of the gland. This stimulation of DNA synthesis was explained by an apparent precocious entry of cells into the MPS. The possibility remains that some of the induced DNA synthesis is due to a small amount of DNA repair that is not detectable. Autoradiographic studies showed that stimulation of DNA synthesis also occurred in glands irradiated at the end of larval development immediately after irradiation. However, this stimulation occurred only after exposure of glands to the higher doses (5000 and 10,000 R), and is taken as a possible sign of DNA repair although one cannot rule out the possibility that the increased synthesis is of a semiconservative nature.

The short term effect of X-irradiation on DNA synthesis when glands were irradiated during the MPS is one of depression. This supports the interpretation that increased DNA synthesis in glands irradiated before the MPS is due to precocious DNA synthesis since cells in the MPS have already entered the synthetic phase. The long term effect of X-irradiation on DNA synthesis when glands were irradiated before or during the MPS is clearly one of depression. Depression of DNA synthesis was explained on the basis of observations in mammalian cells that ionizing radiation alters various cellular processes associated with DNA synthesis. In addition, the long term depressive effect was accounted for by an X-ray induced prolongation of the larval period which implies a slowing down in the

rate of DNA synthesis in all tissues including the salivary glands. The mechanisms for precocious DNA synthesis, repair of DNA damage and depression of DNA synthesis are discussed.

Effects of X-irradiation on DNA Synthesis in Salivary Gland Cells of *Drosophila hydei*

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INTRODUCTION

Stimulation of DNA synthesis within an hour or two after exposure to high doses of ionizing radiation has been observed in some insects. Pavan and Basile (1964) found that high doses of gamma-irradiation (up to 20,000 R) stimulated DNA synthesis in polytene chromosomes of *Rhynchosciara angelae* (Diptera, Sciaridae). Stimulation of DNA synthesis has also been found after high doses of X-irradiation in grasshopper neuroblasts (McGrath, 1963) and in polytene chromosomes of *Drosophila melanogaster* (Diptera, Drosophilidae) (Plaut and Valencia, 1969). These findings were unexpected considering known effects of ionizing radiation on cellular processes in mammalian cells. High doses of ionizing radiation have been found to (1) uncouple oxidative phosphorylation (Van Bekkum, 1957; Creasy and Stocken, 1959), (2) inhibit enzyme synthesis (Bollum *et al.*, 1960), (3) induce release of degradative enzymes from lysosomes (Wills and Wilkinson, 1966), and (4) induce formation of strong oxidizing agents such as lipid peroxides (Willis and Rotblat, 1964) in various tissues of the rat. Any one of these major disturbances in cellular metabolism could inhibit DNA synthesis in insect cells, but it is possible that such disturbances in cellular processes may not be evident until a few hours

or longer after irradiation.

The present investigation was undertaken to study the immediate and long term effects of X-irradiation on DNA synthesis in polytene chromosomes from salivary gland cells of Drosophila hydei. Unlike most cell populations, larval salivary gland cells of Drosophila do not divide. Thus it is possible to study the long term effects of high doses of radiation on DNA synthesis independent of any effects on mitotic progression.

The present findings corroborate those of Pavan and other workers mentioned above that there are short term stimulatory effects of radiation on DNA synthesis. In addition, the present findings suggest that stimulation of DNA synthesis by X-irradiation is of two types: (1) precocious DNA synthesis of cells irradiated just before the DNA synthetic phase, as measured by liquid scintillation counting and autoradiography, and (2) DNA repair like that previously reported by McGrath (1963, 1964) and Plaut and Valencia (1968) in cells irradiated at times when no DNA synthesis is normally observed. The present results also clearly indicate that the long term and overriding effect of massive doses of X-irradiation is one of inhibition of DNA synthesis. The mechanisms for precocious DNA synthesis, DNA repair and inhibition of DNA synthesis after X-irradiation are discussed.

LITERATURE REVIEW

Increasing evidence that DNA is the most important macromolecular target for radiation damage to living systems has prompted radiobiologists to study the effects of radiation on DNA synthesis. In the past 20 years, a great deal of attention has been focused on the effects of ionizing radiation on DNA synthesis for three major reasons: The first one is related to cancer induction and therapy — a better understanding of the action of ionizing radiation on DNA synthesis as well as on other cellular processes is obviously desirable. The second reason is the hope that the understanding of the interaction between a physical agent and living cells will give information on the cellular events leading to the biosynthesis of DNA. The third reason is simply that medical and industrial workers as well as patients are often exposed to ionizing radiation. It is only reasonable that one should find out how radiation affects living cells.

Depression of DNA synthesis after exposure to ionizing radiation has been reported in a great number of biological systems. The literature on the subject has been extensively reviewed by several workers (Holmes, 1957; Kelly, 1957; Stocken, 1959; Goutier, 1961). With the discovery of DNA repair in bacteria after UV (ultraviolet) irradiation (Pettijohn and Hanawalt, 1963, 1964), workers began to look for stimulation of DNA synthesis by irradiation in higher organisms as an indication of DNA repair. The search for radiation

induced unscheduled DNA synthesis (synthesis in the pre- or post-synthetic period) has been intensive in the last five years. It has since been found in many different types of organisms and cultured mammalian cells. However, it has not always been easy to correlate unscheduled synthesis with DNA repair. In some cases, unscheduled synthesis has been explained as precocious semiconservative replication; in others, as repair replication. The insertion of precursors into parental DNA strands as determined by equilibrium density gradient centrifugation (nonconservative replication) is interpreted as repair or "patching" of DNA strands after the damaged sections have been excised.

Depression of DNA Synthesis

The evidence now available clearly establishes that relatively high doses of ionizing radiation inhibit DNA synthesis and lower doses delay it without inhibiting it very much, provided they are given before the onset of synthesis. DNA synthesis has usually been followed by measuring the incorporation of various precursors into DNA and a decreased rate of incorporation has been interpreted as evidence for inhibition of DNA synthesis.

In Vivo Studies

The effects of X-irradiation on DNA synthesis in *in vivo* systems

of the rat and mouse have been extensively studied. In many cases, DNA synthesis was measured several hours or even days after whole body irradiation and inhibition was always observed. In the regenerating liver of the rat, the rate of ^{32}P incorporation into DNA was depressed a few hours after 800 R (roentgens) of X-irradiation (Kelly et al., 1957). In the Ehrlich ascites tumor of the mouse, a 35% decrease in the rate of ^{32}P incorporation into DNA was found three hours after 1000 R of X-irradiation (Harrington et al., 1957). In spleen of rats given 325, 500, and 700 R, Mandel and Chambon (1959) found a 70% decrease in the incorporation rate of ^{32}P into DNA 2 1/2 days after irradiation. These results represent extended observations of in vivo effects of X-irradiation on DNA synthesis and are inconclusive as evidence for "direct" effects on the DNA-synthesizing mechanism once cell deaths and changes in cell population are taken into consideration.

Several workers (Holmes, 1957; Stocken, 1959; Goutier, 1960) have emphasized the importance of taking into account cell deaths and changes in the cell population when DNA synthesis is studied later than two to three hours after irradiation. It seems highly likely that at times later than two hours after exposure, dying or dead cells are present, especially in radiosensitive tissues (Trowell, 1955, 1965). Healthy cells that recolonize the damaged tissue after the cell debris has been cleared away have often been found to be different from the

cells initially present before irradiation. Rat lymph node cells reappearing a few days after whole body irradiation were shown to have a larger nucleus, a lower DNA content, and a lower rate of ^{32}P incorporation into DNA than normal cells (Biagini et al., 1958). Cooper (1959) found that 300 R given to a rat caused a decrease in the percentage of large lymphocytes in the lymph but enhanced the proportion of those lymphocytes synthesizing DNA within a few days after irradiation. Such changes in the cell population and/or cell deaths alone could account for inhibition of DNA synthesis.

Some workers have tried to avoid the complicating effects of cell deaths and changes in cell population on DNA synthesis by making observations of in vivo effects of X-irradiation less than one hour or 1/2 hour after irradiation. The rate of ^{32}P incorporation into rat thymus DNA was depressed by 50%, three minutes after 1000 R of whole body X-irradiation (Ord and Stocken, 1956). Within one hour after 100-800 R whole body irradiation of rats, Nygaard and Potter (1959) found a dose dependent depression in the rate of ^{14}C-thymidine incorporation into thymus and spleen DNA. In the small intestine, 200-800 R also produced a similar depression within the first hour. Thus it appears that the immediate effect of X-irradiation on in vivo DNA synthesis is also one of inhibition. The same workers also found stimulation of DNA synthesis in the small intestine and spleen of the rat after 50-100 R of X-irradiation. The significance of this finding

will be discussed in a later section.

Abscopal Effects

Putting aside cell deaths and changes in cell population, investigators were still faced with the problem of "abscopal" effects when the effect of radiation on DNA synthesis was studied in vivo. Abscopal effects are defined as effects observed in one tissue of an animal when other tissue or tissues of the same animal is exposed to radiation. Such effects on DNA synthesis have been demonstrated in the rat and mouse with techniques like microbeam irradiation and selective shielding of parts of the animal during irradiation.

The pioneer studies of De Hevesy (1945) showed that in a rat bearing two tumors, irradiating one of them altered the ^{32}P uptake by the second. Similar studies showed that DNA synthesis was decreased by 75% in the irradiated tumor and by 50% in the protected one. A drop in DNA turnover in a rat mammary tumor was observed after selective irradiation of liver or muscle (Kelly and Jones, 1950). Harrington and co-workers (1957) studied the ^{32}P incorporation into mouse Ehrlich tumor DNA. Depression of ^{32}P uptake was observed only if (1) irradiation and incubation took place in vivo or (2) if cells, incubated in vitro with ^{32}P, were irradiated before being injected into previously irradiated mice. No depression of incorporation was observed if (1) the tumor from a whole body irradiated animal was

incubated in vitro with ^{32}P, or if (2) tumor cells were irradiated and incubated in vitro with ^{32}P, or if (3) normal tumor cells previously incubated with ^{32}P were injected into irradiated mice. These findings show that there are "indirect" effects on DNA synthesis when animals are given whole body irradiation and these must be taken into consideration when interpreting inhibition of DNA synthesis in a particular tissue.

In Vitro Studies

Because it is not easy to resolve the difficulties raised by abscopal effects, attempts have been made to study DNA synthesis in in vitro systems that are sensitive to ionizing radiation and can give sufficient material for biochemical analysis. Such systems have been derived from human bone marrow, chick embryo, monkey kidney, rabbit appendix, and various tissues of the rat and mouse. Studies described in the following paragraph concern short term effects of radiation; DNA synthesis was followed within two to three hours after irradiation.

In human bone marrow cultures, incorporation of ^{14}C-formate into DNA was reduced by 50% after 2000 R of X-irradiation (Lajtha, 1959). In studies of ^{14}C-formate uptake into DNA of bone marrow homogenates and chick embryo suspensions, a 50% reduction was caused by 2400 R of gamma-irradiation in bone marrow and by 5000 to

20,000 R in chick embryo. Incorporation of ^{32}P into DNA of mouse and monkey kidney cells *in vitro* went on for six days at one-half or one-third of the normal rate after 5000 R of X-irradiation (Whitmore *et al.*, 1958). These *in vitro* studies show that X-irradiation can depress DNA synthesis in the absence of abscopal effects or changes in cell population. However, higher doses are required to demonstrate such a "direct" effect.

The conclusion drawn from both *in vivo* and *in vitro* studies is that in a whole body irradiated animal, the changes of many biochemical functions observed in a particular tissue originate in most cases from a mixture of effects at the site of absorption of radiation ("direct" effects), abscopal effects, cell death, and changes in cell population ("indirect" effects).

Biochemical Effects on the DNA-synthesizing Mechanism

After it was established, from *in vitro* studies such as those just described, that the "direct" effect of radiation on DNA synthesis is one of inhibition, workers turned their attention to the effects of radiation on the DNA-synthesizing mechanism. This line of research began with studies on the radiosensitivity of DNA synthesis at different stages of the mitotic cycle.

It has been noticed by many workers that DNA synthesis is depressed by much lower doses of X-irradiation when the cells are

irradiated during the presynthetic (G_1) period than during the synthetic (S) period in the mitotic cycle. Lajtha and co-workers (1958) measured the incorporation rate of ^{14}C-formate into DNA of human bone-marrow cultures by autoradiography. It was found that 200-300 R of gamma-irradiation given to cells in S did not depress or delay DNA synthesis. However, the same dose given during G_1 delayed synthesis in about half of the cells. ^{3}H-thymidine incorporation into DNA of fibroblasts *in vitro* was markedly depressed only if irradiation was given when the majority of cells were in the G_1 period (Mandel *et al.*, 1956). In the regenerating rat liver after partial hepatectomy, Beltz *et al.* (1957) found that 375 R of X-rays depressed the incorporation of orotic acid into DNA; a dose of 1500 R abolished it. When cells were irradiated in the S period, the effect of 1500 R was barely perceptible. Similar observations were made by Kelly *et al.* (1957) when regeneration of the liver was induced by carbon tetrachloride (CCl_4) intoxication. The evidence seems conclusive that DNA synthesis is comparatively more resistant to ionizing radiation in the S period than in G_1.

Some workers attempted to study the effect of radiation on biochemical events involving DNA synthesis in relation to the mitotic cycle (Bollum *et al.*, 1960). Others studied various biochemical alterations after irradiation that might be used to explain depression of DNA synthesis. These biochemical studies were done using mostly

mammalian systems both in vivo and in vitro, and will be described under the general headings of (1) effects on precursor pool size, (2) effects on enzyme synthesis and activity, (3) effects on oxidative phosphorylation, (4) effects on membrane structure and function, and (5) formation of organic peroxides.

Effects on precursor pool size. It has been suggested that the decreased incorporation of ^{3}H-thymidine into DNA after irradiation is due to an expansion of nucleoside and nucleotide pools, and not an actual decrease in DNA synthesis (Hell et al., 1960; Kelly, 1961; Smets, 1966a, 1966b). Expansion of precursor pool size has been explained by DNA degradation. However, Adelstein and Manasek (1967a), who studied the kinetics of thymidine uptake in spleenic lymphocyte suspensions of the golden hamster, could find no change in the endogenous thymidine pool shortly after 15 krads of X-irradiation. It was shown in the same system that the decrease in incorporation rate of ^{3}H-thymidine after irradiation was due to a specific inhibition in the utilization of thymidine and other pyrimidine nucleosides for DNA synthesis (Adelstein and Manasek, 1967b). Painter and co-workers (1968) found that very little degradation occurred in DNA of cultured mammalian cells after high doses of UV and X-irradiation. This is in direct contrast to the situation in bacteria in which DNA degradation after irradiation, especially X-irradiation, is extensive (Emmerson and Howard-Flanders, 1965).

Thus the evidence in mammalian systems has not supported the hypothesis that depression of DNA synthesis is due to an expansion of the precursor pool size.

Effects on enzyme synthesis and activity. The DNA-synthesizing enzyme was discovered by Kornberg and co-workers (Lehman, Bessman, Simms and Kornberg, 1958). The effects of ionizing radiation on this enzyme system was studied in the regenerating rat liver (Bollum et al., 1960; Lehnert and Okada, 1966) and in the rat kidney (Main et al., 1963). Bollum and co-workers induced regeneration in the rat liver by partial hepatectomy. Regenerating cells were quite well synchronized. DNA synthesis occurred from the 18th to the 30th hour after operation with a maximum at the 27th hour. The mitotic index increased considerably and reached a maximum at about 30 hours. It was found that 375, 750, and 1500 R of whole body X-irradiation given six hours after operation completely inhibited the increase in thymidine kinase and DNA polymerase levels that normally occurred between six and 24 hours. The same doses given 16 hours after operation, a time previously shown to completely block DNA synthesis in regenerating liver in vivo, did not prevent the usual increase in enzyme levels between 16 and 24 hours. Bollum concluded that X-irradiation given in the early part of the G_1 period somehow prevented the synthesis of enzymes necessary for DNA synthesis; once formed, the enzymes were radioresistant. However, he did not attempt to explain why DNA

synthesis was completely blocked when irradiation was given 16 hours after operation. In the same system, the rate of DNA synthesis in the S stage cells in vivo was found to be related to the level of thymidine-kinase-DNA-polymerase estimated in in vitro studies (Lehnert and Okada, 1966).

It has been found that in rat, whole body X-irradiation (840 rads) given between 10 minutes to 18 hours after uninephrectomy prevented the appearance of thymidine-kinase-DNA-polymerase in the remaining kidney (Main et al., 1963). When irradiation was given between 19 and 24 hours, considerable enzyme activity was observed. Main and co-workers concluded that the induction of the enzyme system necessary for DNA synthesis is highly radiosensitive. This is in exact agreement with Bollum's conclusion. In both studies, no explanation was given as to how X-rays might prevent enzyme synthesis.

Effects on oxidative phosphorylation. The importance of oxidative phosphorylation in DNA synthesis is obvious since adenosine triphosphate (ATP) is a necessary component in the DNA-synthesizing system as well as the major source of energy supply for cellular processes. Changes in ATP production after irradiation have been studied in isolated mitochrondria and in isolated nuclei.

It has been demonstrated that the rate of oxidative phosphorylation in isolated thymus mitochondria of the rat is decreased after moderate to high doses of whole body X-irradiation (Van Bekkum and

Vos, 1955; Van Bekkum, 1957). In isolated spleen mitochondria given 100 R of whole body X-irradiation, the efficiency of oxidative phosphorylation was decreased by 50% (Van Bekkum, 1957). Similar observations were made in mitochondria isolated from rat liver (Clark, 1955; Clarke and Lang, 1965), but in these cases doses of up to 5000 to 10,000 rads of gamma- and electron-irradiation (from a 15 Mev linear accelerator) were required to achieve the same effect. The same mitochondria irradiated in vitro within the electron dose range of 0-100 krads showed a 10-40% decrease in efficiency depending on the substrate studied (Clark, 1967). These data indicate a partial uncoupling of mitochondrial oxidative phosphorylation. Clark postulated that radiation disrupts the delicate organization associated with the membrane structure of the mitochondria, the intergrity of which was known to be essential for efficient oxidative phosphorylation (Lehinger, 1964; Wills and Wilkinson, 1966).

A decrease in ATP synthesis in isolated nuclei of rat tissues has also been observed. Nuclear oxidative phosphorylation was found to occur only in radiosensitive tissues of the rat — spleen, thymus, lymph node, bone marrow, and intestinal mucosa, but not in radioresistant tissues — brain, liver, kidney and pancreas (Creasy and Stocken, 1959). After 25-100 R of whole body X-irradiation, oxidative phosphorylation was abolished in nuclei prepared from the radiosensitive tissues. It has been shown that the nuclear fraction of rat thymus

contained about 59% of the cellular ATP (Betel and Klouwen, 1967). Under anaerobic conditions, nuclear ATP is degraded. When, after an anaerobic incubation, the nuclei are incubated under aerobic conditions, endogenous nuclear ATP is re-synthesized. Such re-synthesis is markedly inhibited after low doses of whole body X-irradiation (Klouwen, 1965). Similar observations were made in myeloid leukemic cells and C57BL-lymphoma-cells of the rat (Klouwen and Appelman, 1967). It has been demonstrated that the decreased re-synthesis of ATP is caused by a decline in the adenine-nucleotide content of the nuclei as an effect of X-irradiation (Betel, 1967). It is noteworthy that higher radiation doses are required to inhibit oxidative phosphorylation in the nucleus to the same extent as in the mitochondria. Since ATP is vital for the polymerization of bases in DNA synthesis, nuclear oxidative phosphorylation may be a vital target for radiation damage.

Effects on membrane structure and function. The integrity of the cell as well as its many processes is dependent on the structure and function of membranes. The effect of radiation on membranes has been studied in mammalian systems and can correlate with alterations in various cellular processes after irradiation.

It has been demonstrated in different mammalian cells exposed to high doses of X- and gamma-irradiation (3000-5000 rads) that plasma membranes are ruptured and mitochondria are disorganized (Bacq and

Alexander, 1961; Casarett, 1968). These findings indicate direct damage to the membranes and altered permeability. In rat liver mitochondria irradiated in vitro, electron doses (from linear accelerator) up to five krads were found to inhibit active transport of potassium and calcium ions, as determined by measuring the content of the two ions after irradiation (Wills, 1966a). Wills suggested that the effect on ion transport is a direct result of damage to the mitochondrial membrane. Exposure of lysosomes isolated from the rat liver to 100 krads of electron irradiation in vitro resulted in a release of hydrolytic enzymes from these cell components (Wills and Wilkinson, 1966). This has been taken as evidence for an increase in the permeability of the lysosomal membrane as a result of radiation damage.

The mechanism of radiation damage to cell membranes has been studied in relation to the formation of lipid peroxides after irradiation. Lipids extracted from liver, heart, spleen and kidney of the rat form peroxides when irradiated as aqueous emulsions in vitro (Wills and Rotblat, 1964; Wills, 1966b). Isolated nuclei, mitochondria, lysosomes and microsomes of the rat all form lipid peroxides after in vitro irradiation with electron doses of 2-100 krads (Wills and Wilkinson, 1967a). The relative amount of peroxide formed in each subcellular fraction, after identical conditions of irradiation and incubation, is microsomes > lysosomes > mitochondria > nuclei. The

precise sites of peroxide formation are not known. Phospholipids containing unsaturated fatty acids are known to be essential components of most, if not all, extracellular and intracellular membranes (Van Deenan, 1965). Oxidation of these fatty acids to form peroxide has been shown to cause membrane destruction and leakage of hemoglobin from rat liver erythrocytes (Tsen and Collier, 1960). Release of hydrolytic enzymes from lysosomes after irradiation has been associated with peroxide formation in the lysosome membrane (Wills and Wilkinson, 1966). These results indicate that radiation damage to membrane structure and function may be mediated through the formation of lipid peroxides.

DNA synthesis may be affected by alterations in membrane structure and function in many ways. Changes in the permeability of membranes may affect nucleotide pools. Alterations in the mitochondrial membrane may affect ATP production which in turn may affect DNA synthesis. However, since relatively large doses of radiation are required to demonstrate effects on membranes, it is difficult to explain inhibition of DNA synthesis after low doses of irradiation by damage to membrane structure and function.

Formation of organic peroxides. Formation of organic peroxides such as lipid peroxides has been demonstrated in various cells after moderate doses of whole body irradiation (Bacq and Alexander, 1961). These peroxides have been found to be extremely toxic to rats (Hogan

and Philpot, 1957), and the amount required to kill a rat is of the same order as that produced by a lethal dose of X-irradiation. Also injection of an enzyme, lipoxidase, which catalyses peroxide formation, produces a radiation-like death in rats (Muset et al., 1959). However, the symptoms of death are different from those of radiation. It is conceivable that these radiomimetic properties of organic peroxides may also affect DNA synthesis.

Lipid peroxides formed after irradiation have been shown to be powerful oxidizing agents and to attack —SH proteins (Wills, 1961; Lewis and Wills, 1962; Wills and Wilkinson, 1967b). It has been shown that DNA polymerase of bacteria contains a single sulfhydryl group (Englund et al., 1968). Oxidation of this group, if it also exists in DNA polymerase of mammalian cells, may affect enzymatic activity leading to an inhibition of DNA synthesis.

In conclusion, any or all or a mixture of the biochemical events described so far can be used to explain inhibition of DNA synthesis induced by ionizing radiations. In any case, it is difficult to restrict the effects of radiation to any particular biochemical event or events. X-irradiation causes derangement of interdependent cellular processes.

Stimulation of DNA Synthesis

The first indication that X-rays might stimulate DNA synthesis

was found in the spleen and small intestine of the rat. Incorporation of ^{14}C-thymidine into the DNA of these tissues was increased during the first hour after 50 and 100 R of X-irradiation (Nygaard and Potter, 1959). No attempt was made to explain this increase. Workers at that time were predominantly occupied with depression of DNA synthesis by ionizing radiation. Das and Alfert (1961) reported such a stimulation in onion root meristem during high doses of X-irradiation. In grasshopper neuroblasts, an increase in ^{3}H-thymidine incorporation into DNA has been found when cells were exposed to high doses of X-rays at times in the cell cycle not normally associated with DNA synthesis (McGrath, 1963).

In synchronized cultures of the slime mould Physarum polycephalum, a 40% increase in the specific activity of DNA was observed after 25,000 R of X-irradiation (McGrath and Setlow, 1964). In this organism, DNA synthesis (S) occurs during the first three hours after metaphase. When irradiation was given at the end of the S period, the increase in specific activity of DNA occurred during or at the beginning of the next S period. McGrath postulated that the increase in specific activity of DNA might have resulted from a change in the specific activity of the intracellular pool of thymidine as a result of irradiation, or it might reflect repair of damaged DNA. If the second explanation is correct, one would also have to postulate that repair of DNA can only occur during or at the beginning of the S period, since

these are the times when the increase in specific activity of the irradiated DNA was observed.

Repair of DNA Damage

Stimulation of DNA synthesis by irradiation in cultured cells during the non-synthetic periods (unscheduled synthesis) has been extensively studied and has usually been attributed to DNA repair. The mechanism of DNA repair was first studied in bacterial systems. It has been postulated that repair of radiation induced DNA damage in bacteria and other cellular systems involves excision of defective single-strand regions followed by replacement with short stretch of oligonucleotide using the complementary base pairing information in the intact strand (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). This mode of repair has been called repair replication or the "cut-and-patch" hypothesis. The first indication for this mode of DNA repair came from studies in Escherichia coli after UV-irradiation. It has been demonstrated that UV-induced thymine dimers are released from the DNA of certain UV resistant strains of this bacterium in vivo (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). Direct physical evidence for this mode of DNA repair in E. coli came from the studies of Pettijohn and Hanawalt (1963, 1964) as will be described below.

Repair replication in bacteria. Repair replication has been

demonstrated in E. coli after UV-irradiation (Pettijohn and Hanawalt, 1963, 1964) using the technique of equilibrium density gradient centrifugation, essentially the same as that used by Meselson and Stahl (1958) to demonstrate that DNA normally replicates semiconservatively. DNA replication was followed by the use of the thymine analog, 5-bromo-uracil (5BU), as a density label. Cells were allowed to undergo partial DNA replication after UV-irradiation in the presence of 5BU. Chromosomes (DNA) were fragmented, and centrifuged in a cesium chloride density gradient. Following this procedure, a band of density intermediate between that of normal DNA fragments (unreplicated parental DNA) and hybrid DNA fragments (one parental thymine-containing strand and one daughter 5BU-containing strand) was observed. The presence of small amounts of 5BU in DNA fragments giving rise to the intermediate band was demonstrated by denaturing these fragments and rebanding in a cesium chloride density gradient. An intermediate density band between that of parental and daughter strands was observed. These findings indicate that 5BU has been incorporated into parental DNA strands after UV-irradiation, and is taken as evidence for the patching step in the "cut-and-patch" hypothesis of DNA repair. Since repair replication involves removal and replacement of parental DNA strands without a net increase in the amount of DNA, it has also been termed nonconservative replication. Repair replication has also been demonstrated in other bacteria

(see review by Hanawalt, 1968).

It has been postulated that the "cut-and-patch" mode of DNA repair involves two types of enzymes. The first of these are known as nucleases, the "cut" enzymes, which seemingly produce single-strand breaks in damaged regions of DNA and remove damaged segments. Such nucleases have been demonstrated in Micrococcus lysodeikticus. An endonuclease and an exonuclease have been partially purified from extracts of this bacterium (Takagi et al., 1968; Grossman et al., 1968). The endonuclease is specifically active on UV-irradiated DNA and has been shown to induce only single-strand scissions. In the presence of this enzyme alone, no thymine dimers are released from UV-irradiated DNA; however, in combination with the exonuclease, release of thymine is observed. Released thymine dimers have been found in short oligonucleotides (four to five nucleotides). These findings indicate that the endonuclease found in M. lysodeikticus extracts induces single-strand breaks in DNA at points close to thymine dimers; the free ends produced are susceptible to attack by the exonuclease which then removes nucleotide fragments containing thymine dimers.

The second type of enzymes, the "patch" enzymes, apparently function in a manner similar to DNA polymerase: they presumable replace the single-strand gap produced by the "cut" enzymes with nucleotides using the base pairing information in the intact

complementary strand. Although these enzymes have not yet been found, the evidence so far seems to support the "cut-and-patch" mode of DNA repair, at least in the repair of UV-induced thymine dimers (Hanawalt et al., 1968).

There is now ample evidence that the "cut-and-patch" mode of repair of thymine dimers after UV-irradiation is also used to repair DNA damage in bacteria resulting from X-irradiation and treatment of various alkylating agents and mutagens. Repair replication has been observed in E. coli after X-irradiation (Howard-Flanders and Boyce, 1966). Furthermore, it has been shown in mutants of E. coli that show little or no repair replication after X-irradiation are also incapable of repairing UV-induced thymine dimers. This is also true in X-ray sensitive mutants of Haemophilus influenzae (Setlow et al., 1968).

Repair replication has also been observed in E. coli (Hanawalt and Haynes, 1965; Cerda-Olmedo and Hanawalt, 1967), in Bacillus subtilus (Strauss et al., 1968) and in Micrococcus lysodeikticus (Strauss and Robbins, 1968) after treatment with alkylating and mutagenic agents. Mutants of all three bacteria that are defective in repair replication after treatment with these agents have also been found to be defective in the repair of UV-induced thymine dimers. These findings indicate that the mechanism for the repair of UV-induced thymine dimers is apparently also used to repair DNA damage induced

by X-irradiation and treatment with alkylating and mutagenic agents. It has been suggested by Hanawalt (1968)

> . . . that it may not be the precise nature of the base damage that is recognized but rather some associated secondary structural alterations in the phosphodiester backbone of the DNA; the damage recognition step may be formally equivalent to threading the DNA through a close-fitting sleeve that gauges the closeness-of-fit to the Watson-Crick structure.

Repair replication and unscheduled DNA synthesis in mammalian cells. Studies of replication repair in bacteria have been extended to mammalian cells. Using the same technique of equilibrium density gradient centrifugation, repair replication has been demonstrated in a number of human and rodent cell types. In HeLa cells, repair replication was observed after exposure to UV- and X-irradiation (Cleaver and Painter, 1968; Painter and Cleaver, 1967). Similar results were obtained in cultured Chinese hamster cells (Cleaver and Painter, 1969). Repair replication has also been observed in the following cell types after UV-irradiation: human WI-38[a], primary human skin (normal), human leukocytes, mouse L and primary mouse fetal (see review by Painter and Cleaver, 1969). Occurrence of repair replication after X-irradiation has not yet been tested in these cell types. The evidence seems conclusive that like bacteria, mammalian cells are also capable of repairing UV and X-ray induced DNA damage.

The "cut-and-patch" hypothesis of DNA repair in bacteria has also been used to explain DNA repair in mammalian cells after UV- and

X-irradiation (Painter, 1968; Dalrymple et al., 1968). However, as has been pointed out by Painter and Cleaver (1969), this model runs into difficulties in the case of mouse L cells (Klimek, 1965) and Chinese hamster cells (Trosko, Chu and Carrier, 1965) in which repair replication has been demonstrated after UV-irradiation although neither cell type shows any apparent excision of thymine dimers. Thus the mechanism for DNA repair in mammalian cells may be different from that found in bacteria.

Another approach to the study of DNA repair in mammalian cells is the demonstration of unscheduled DNA synthesis (synthesis outside S) after UV- and X-irradiation. This phenomenon together with studies on repair replication in the same cell types, has been summarized by Painter and Cleaver (1969). They noted that unscheduled DNA synthesis and repair replication always occurred together. In addition, at least in the case of mouse L and Chinese hamster cells, there is a rough correlation of the extent of one with that of the other. In cells derived from patients with xeroderma pigmentosum, which do not show repair replication after UV-irradiation, no unscheduled DNA synthesis has been observed either (this is the first instance in which cancer has been correlated to DNA repair in mammals). Painter and Cleaver concluded that the two phenomena reflect the same primary process of DNA repair.

Although most workers interpret unscheduled DNA synthesis as

DNA repair, this phenomenon can sometimes be more readily explained by precocious DNA synthesis. Unscheduled DNA synthesis has been observed in HeLa cells after exposure to 500 and 1000 R of X-irradiation (Feinendegen et al., 1968). The majority of cells showing DNA label have been shown to be in the latter part of the G_1 period at the time of irradiation. This X-ray induced DNA synthesis is believed to result from precocious entry of late G_1 cells into the normal S period and/or DNA repair. Thus one must keep in mind the possibility of precocious DNA synthesis when correlating unscheduled DNA synthesis with DNA repair.

Stimulation of DNA Synthesis in the Dipteran Salivary Gland

Stimulation of DNA synthesis has been reported in polytene chromosomes of Dipteran salivary gland cells, as has been mentioned in the introduction of this thesis. However, there is still no compelling evidence that such stimulation reflects DNA repair. Although Plaut and Valencia (1969) suggested that stimulation of DNA synthesis in salivary gland cells of Drosophila melanogaster is a manifestation of DNA repair, such stimulation can just as well be explained by precocious DNA synthesis, as will be pointed out in the discussion section of this thesis. In the absence of biochemical studies such as those of Painter and Cleaver (1969), it is difficult to distinguish between the two processes.

MATERIALS AND METHODS

The Biological System

The organs studied in the experiments described in this thesis are salivary glands from the third instar larvae of Drosophila hydei. At this stage of development, the glands have attained a length of about 1 mm to 3 mm and can be dissected out with fine watch maker's forceps without difficulty. A gland is made up of two equal lobes and each lobe has between 130-140 cells (Wong, 1969).

In the salivery glands of Drosophila, and apparently of flies in general, cells do not divide from the time the first gland primordium is formed in the embryo. The growth of the gland is then entirely through cell enlargement (Bodenstein, 1950). The chromosome number does not increase as the cell grows (Swift, 1962). This is apparently associated with the unexplained property of fly chromosomes to undergo "somatic pairing," that is, for homologous chromosomes to remain closely associated through interphase. Nevertheless, the DNA undergoes progressive doubling to produce nuclei with 2:4:8:16..... 1,024 times the haploid DNA value, as measured in Drosophila melanogaster by cytophotometric techniques, and may be higher in other Drosophila species (Alfert, 1954; Swift, 1962). Thus the giant or "polytene" chromosomes so formed are probably bundles of up to 1,024 strands or more, each of which may resemble the tiny chromosomal strand found

in a normal diploid cell. DNA synthesis in the gland cells is partially synchronized. The nuclei increase gradually in DNA content in an anterior-posterior direction as larval development progresses (Bodenstein, 1950; Alfert, 1954).

The morphological development of the larval salivary gland in Drosophila melanogaster has been described by Bodenstein (1950) and is similar in Drosophila hydei. The overall rate of larval development in both species is slower in the male than in the female (Wong, 1966, 1967). DNA synthesis in the male salivary gland of Drosophila melanogaster has been shown to lag behind that of the female (Rodman, 1967). Therefore, to promote synchrony of developmental stages, only female salivary glands were used in the present investigation.

Culturing Drosophila hydei

A stock of Drosophila hydei was obtained from the University of Chicago and mass cultured at room temperature under natural light conditions in a standard medium of corn meal, agar, dextrose, sucrose and yeast as described by Lewis (1960). Unless otherwise stated, all animals used in the experiments described here were raised in half-pint milk bottles filled to one quarter full with culturing medium.

Staging of Larvae

As most Drosophila workers know, it is very difficult to

synchronize larval development in this genus. Precision staging of a large number of larvae was done in the following manner.

Fifteen to 20 pairs of adult flies from stock cultures were transferred to fresh medium and kept at a temperature of 23.5 ± 0.5°C in air incubators (Precision Scientific). They were subcultured every week; parent flies were discarded after the third subculture. Newly emerged adults were aged in groups of about 50 in fresh medium for 10 to 15 days at room temperature in natural light. Gravid females, 100 in number, were picked and induced to lay eggs in a specially prepared medium made up of agar, dextrose, sucrose and active dry yeast. This medium was dyed blue with food dye for easier observation of the eggs laid. The females were placed in a quiet corner of the room where they were left undisturbed. Under these conditions, each group of females laid thousands of eggs in a period of four to six hours. The females were then removed and the eggs incubated at 23.5 ± 0.5°C. At this temperature, the majority of first instar larvae will have hatched 28 to 30 hours from the mid-point of oviposition. When larvae first began to appear on the blue medium, they were washed away with Ephrussi-Beadle solution (Ephrussi and Beadle, 1936) and those that hatched within the next five to ten minutes were washed onto a piece of filter paper which was then placed on standard medium in a petri dish. From these, 500 were picked with forceps, transferred to standard medium in a bottle and raised in a precision water bath at 23.5 ± 0.05°C.

From then on, the medium was supplemented each day with a thick suspension of active dry yeast (Fleischman brand). Under these conditions, the entire larval development for females lasts 200 $\pm$ 2 1/2 hours from the time when they hatched as first instar larvae to the prepupae. The second moult occurs at 126 to 128 hours as determined by the presence of shed cuticles on the culturing medium.

X-irradiation

Female third instar larvae were irradiated in groups of 200 in plastic petri dishes (100 mm x 15 mm) filled with a thin layer of standard medium. Prior to irradiation, larvae were taken out of the culturing medium in the bottle, sexed and divided into two groups; one was used for control and the other for irradiation. X-irradiation was done on a therapeutic X-ray unit (General Electric Maxitron 300) at 20 mA, and 300 keV with 0.35 mm aluminum filter. The distance from the target to the petri dish was 50 cm with a uniform dose rate of 660 R/min over the area of the dish, as measured by a Victoreen-R meter. All irradiations were carried out under these settings at a room temperature of 21 to 24^{o}C. The target distance was slightly varied from experiment to experiment in order to obtain the same dose rate of 660 R/min.

Measuring Thymidine Incorporation

Liquid Scintillation Spectrometry

This technique was employed for the quantitative assay of thymidine incorporation into salivary gland cell DNA. For each sample, ten pairs of glands were dissected from larvae within five minutes, rinsed in Ephrussi-Beadle medium, and incubated for 30 minutes at 25 ± 1°C in 0.3 ml of Ephrussi-Beadle medium to which 25 μc/ml of tritiated thymidine (Schwarz Biochemicals, specific activity about 11,000 mc/mmole) were added. The glands were then fixed in Carnoy's fixative (1:3, 100% acetic acid:100% ethanol) for 15 minutes, left overnight in 70% ethanol, treated in cold TCA (5% trichloroacetic acid at 5°C) for one hour followed by two rinses with cold TCA, three rinses with 95% ethanol, and a final rinse with 100% ethanol. They were then digested in 2 ml of 0.5 M ethanolic (70%) potassium hydroxide for one hour at 60°C. All steps after fixation in Carnoy's were carried out in 2 ml test tubes. The resulting solution was colorless. The radioactivity of each sample was assayed in a three-channel Nuclear Chicago liquid scintillation spectrometer in 10 ml of a detection medium (diotol[1]). The background activity was

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[1] Composition of diotol: 50 ml touluene, 500 ml dioxane, 300 ml methanol, 104 gm napthalene, 6.5 gm PPO (2,5-diphenyloxazole), 130 mg POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene). All chemicals are reagent grade.

determined with samples prepared from unlabeled glands. The chemical consistency of samples was checked by counting each sample with a built in external standard, a radium source. The counting efficiency should be the same if each sample has the same chemical make up. The counting efficiency was 10 to 12% as determined by prepared internal standards.

Autoradiography

This technique was used to study the population of cells incorporating thymidine. For each sample, groups of five glands were pulse labeled in the same manner as described above and nuclei were stained with either orcein or by the Feulgen procedure. For orcein staining, each gland was fixed and squashed in 45% acetic acid immediately after pulse labeling on a clean slide with a siliconized coverslip, frozen over dry ice for coverslip removal, rinsed in 45% acetic acid, and stained in aceto-orcein (3% natural orcein in 65% acetic acid) for one minute. This was followed by three rinses with 45% acetic acid and three rinses with water. For Feulgen staining, the glands were fixed in Carnoy's fixative for ten minutes immediately after pulse labeling. The whole gland was then hydrolyzed in 1 N hydrochloric acid for 12 minutes at 60^{o}C and stained in Schiff's reagent for 45 minutes to one hour. Each gland was then squashed and treated in the same manner as for orcein preparations. Finished

slides were stored in water.

Autoradiographs were prepared by coating the prepared slides with Ilford L4 nuclear track emulsion diluted 1:1 with a 0.1% detergent solution at 42°C. All slides were treated in cold TCA for ten minutes, washed well in distilled water and pre-warmed to 42°C prior to coating with emulsion. Coated slides were dried overnight in a test tube rack and stored in plastic slide boxes. After four days exposure, they were developed in D19 (Kodak) for six minutes, rinsed in an acetic acid stop bath, fixed in acid fixer for ten minutes, and rinsed in running water for 20 minutes. Each slide was then dehydrated in an alcohol series, made into permanent mounts with Canadian balsam, and analyzed under bright-field optics. Counting of nuclei was facilitated by projecting each preparation onto a white sheet of paper with a slide specimen projector (Leitz Prado with adaptor) and marking nuclei on the paper.

RESULTS

DNA Synthesis in Salivary Glands

DNA synthesis at successive stages of the third instar salivary glands, as measured by the rate of ^{3}H-thymidine uptake, is shown in Figure 1. ^{3}H-thymidine uptake at each stage was measured by liquid scintillation counting in ten pooled glands. The age of third instar larvae is expressed in hours from the time of hatching of first instar larvae. Measured ^{3}H-thymidine uptake is expressed in counts per minute above background.

A "Major Period of DNA Synthesis," hereafter referred to as the "MPS," occurs between 154 and 164 hours, with a peak at 158 hours, just before the mid-point of third instar development. This is followed by two smaller synthetic peaks at 171 and 181 hours. DNA synthesis before and after the MPS is relatively low.

Effects of X-irradiation on DNA Synthesis

Liquid scintillation counting was used to study the short and long term effects of X-irradiation on DNA synthesis in salivary glands of the third instar larvae. The time course of ^{3}H-thymidine uptake into gland cells was measured after exposure of larvae to 1250, 2500, 5000, and 10,000 R of X-rays. The results are shown in Figures 2 through 9. Each figure represents one experiment. Larvae were

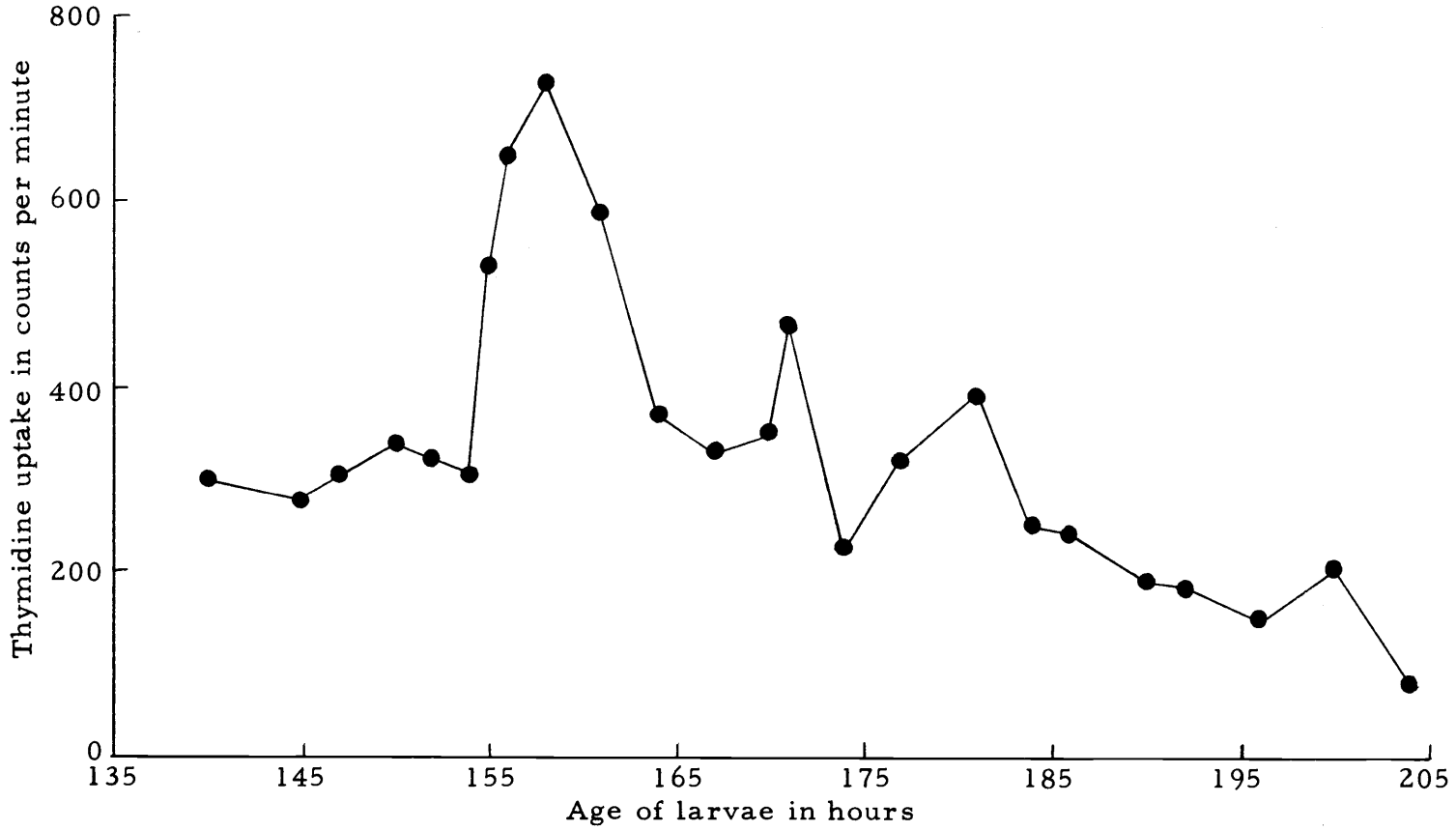


Figure 1. In vitro ^{3}H-thymidine uptake into third instar salivary gland cells of Drosophila hydei as measured by liquid scintillation counting. Thymidine uptake is measured in ten pooled glands and is expressed in counts per minute above background.

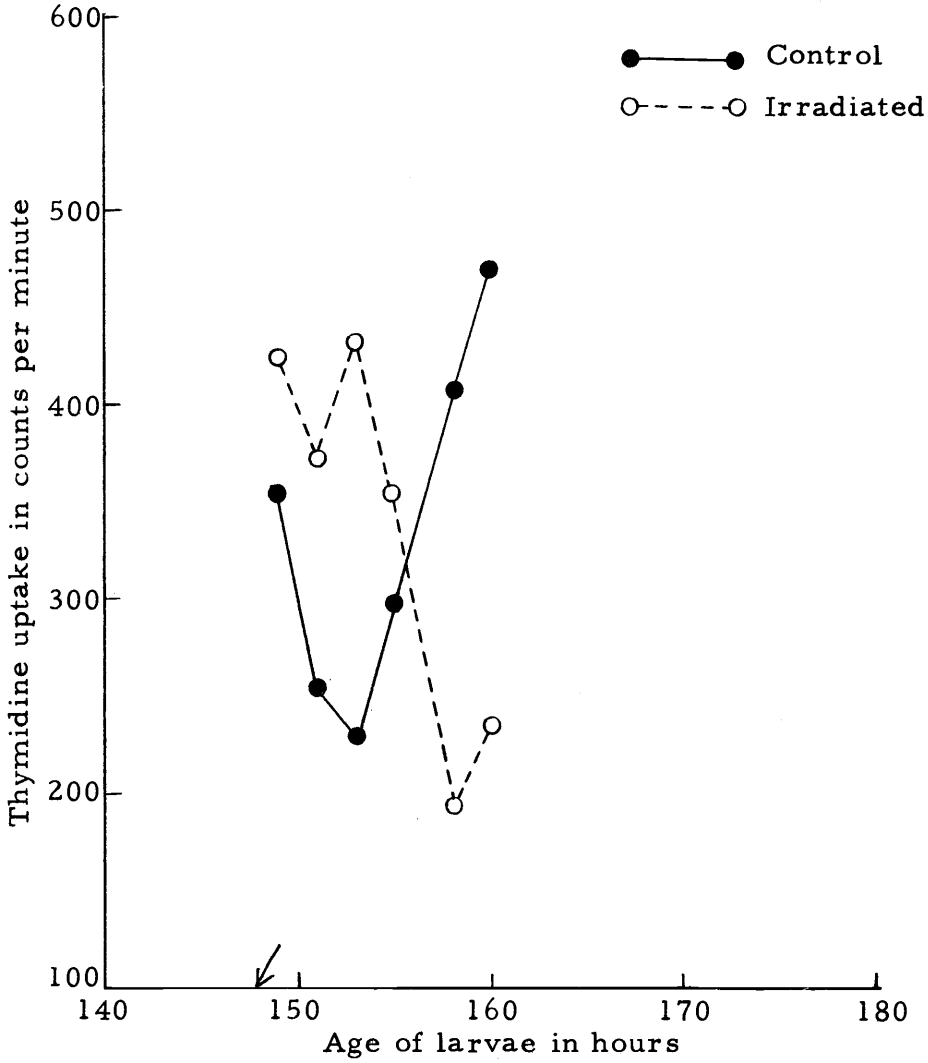


Figure 2. In vitro ^{3}H-thymidine uptake into salivary gland cells after 1250 R of whole body X-irradiation. Arrow indicates time of irradiation (148 hours). Thymidine uptake is measured in ten pooled glands and is expressed in counts per minute above background.

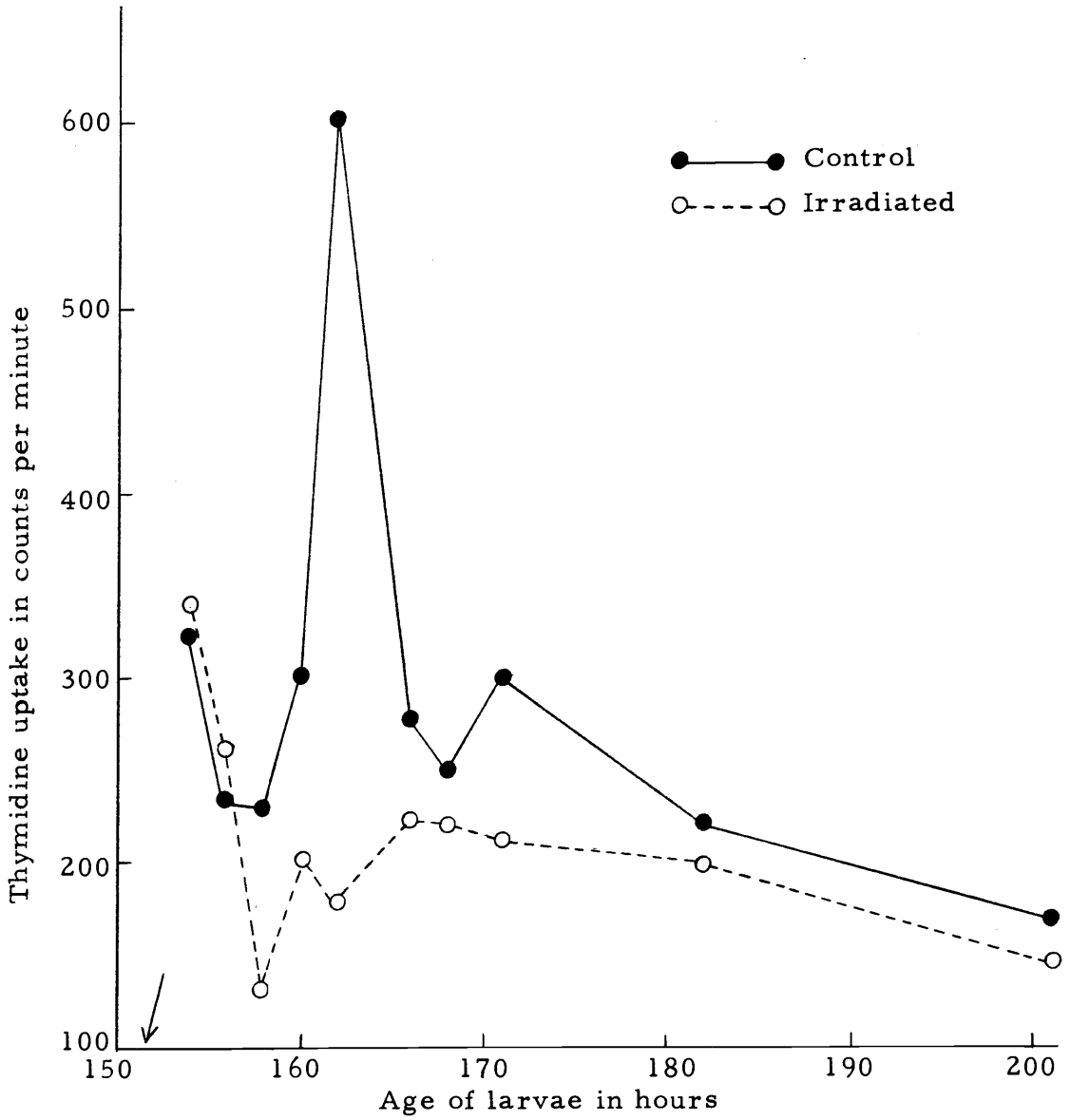


Figure 3. In vitro ^{3}H-thymidine uptake into salivary gland cells after 2500 R of whole body X-irradiation. Arrow indicates time of irradiation (151 hours). Thymidine uptake is measured in ten pooled glands and is expressed in counts per minute above background.

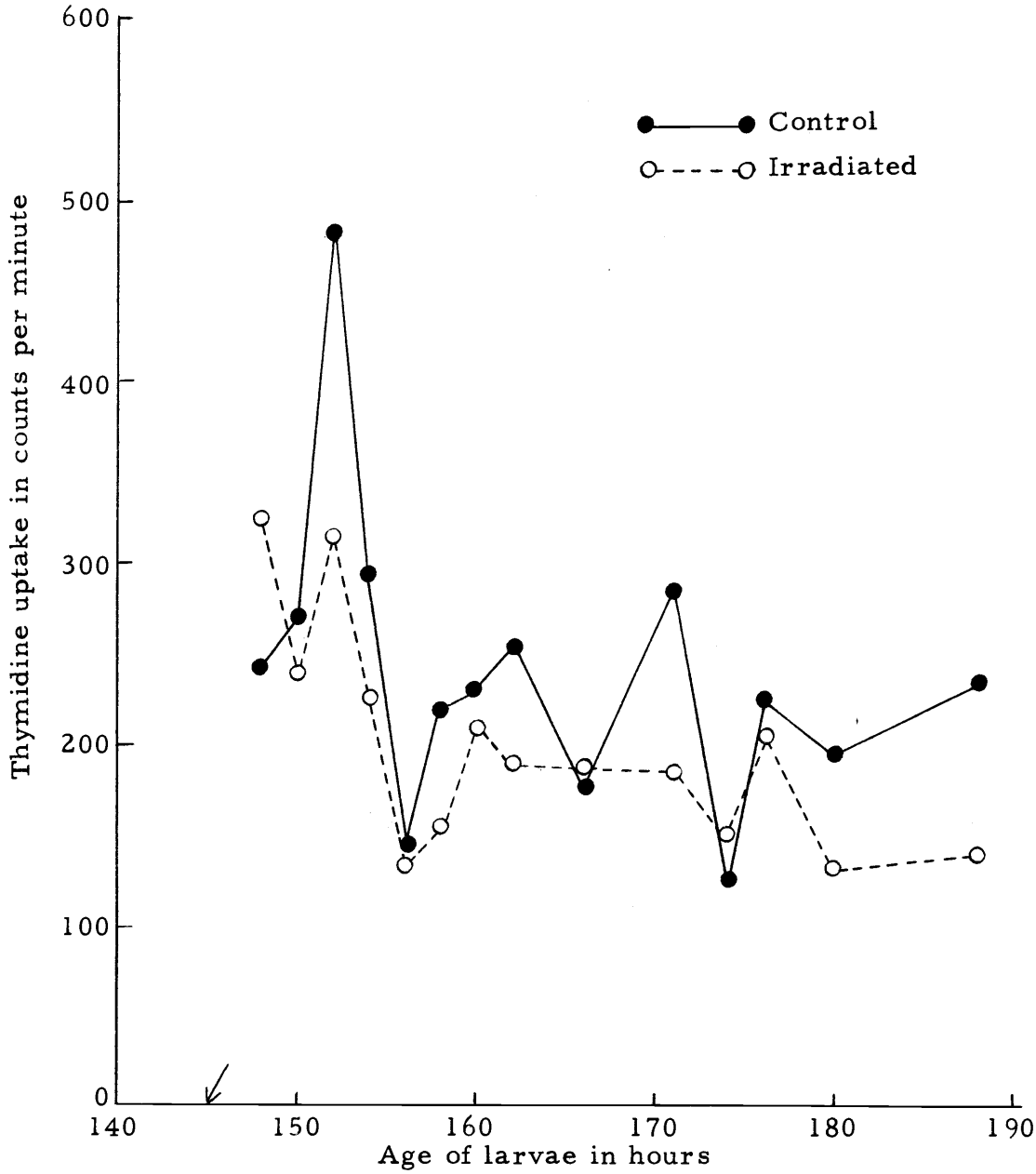


Figure 4. In vitro ^{3}H-thymidine uptake into salivary gland cells after 2500 R of whole body X-irradiation. Arrow indicates time of irradiation (144 hours). Thymidine uptake is measured in ten pooled glands and is expressed in counts per minute above background.

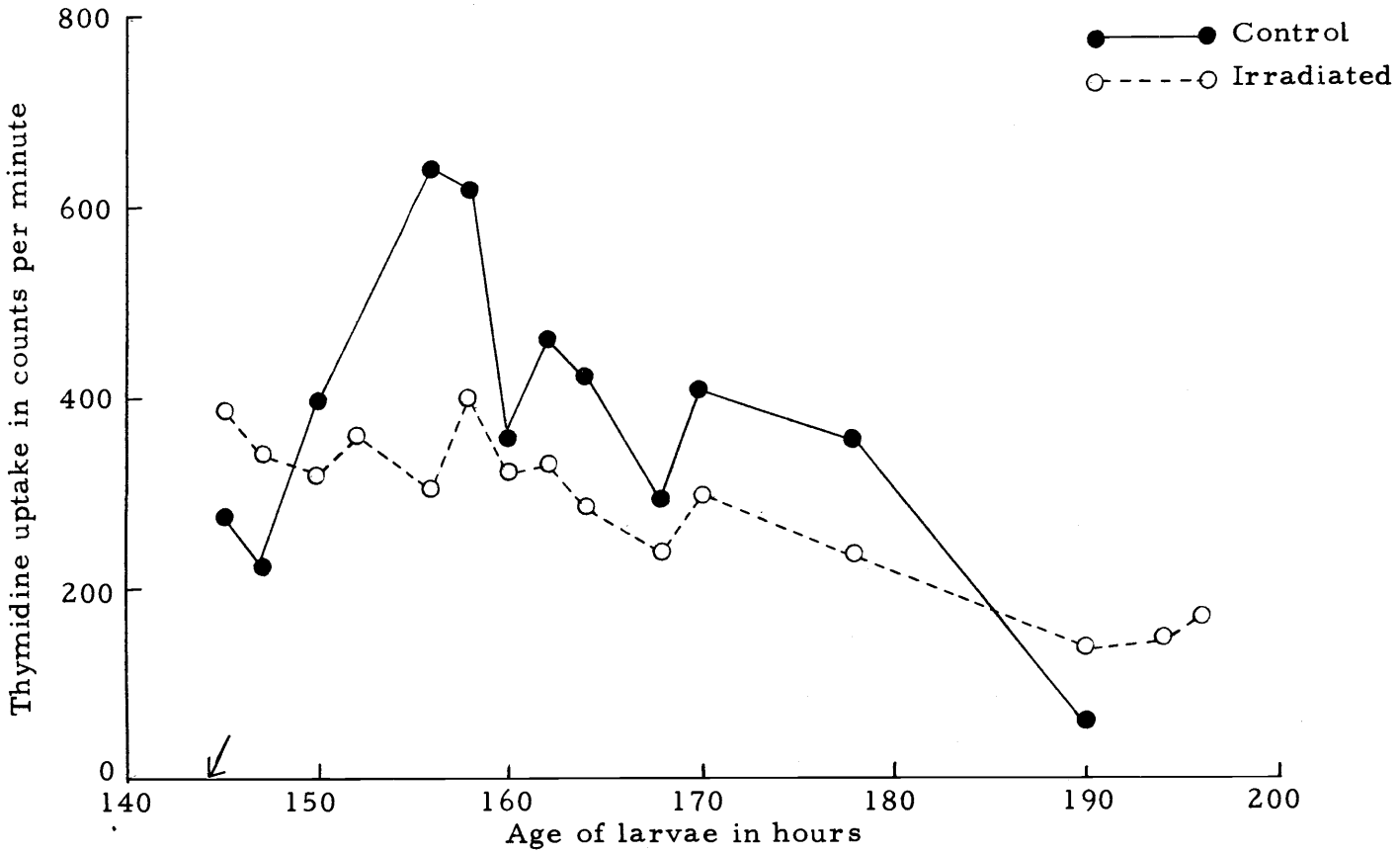


Figure 5. In vitro ^{3}H-thymidine uptake into salivary gland cells after 5000 R of whole body X-irradiation. Arrow indicates time of irradiation (144 hours). Thymidine uptake is measured in ten pooled glands and is expressed in counts per minute above background.

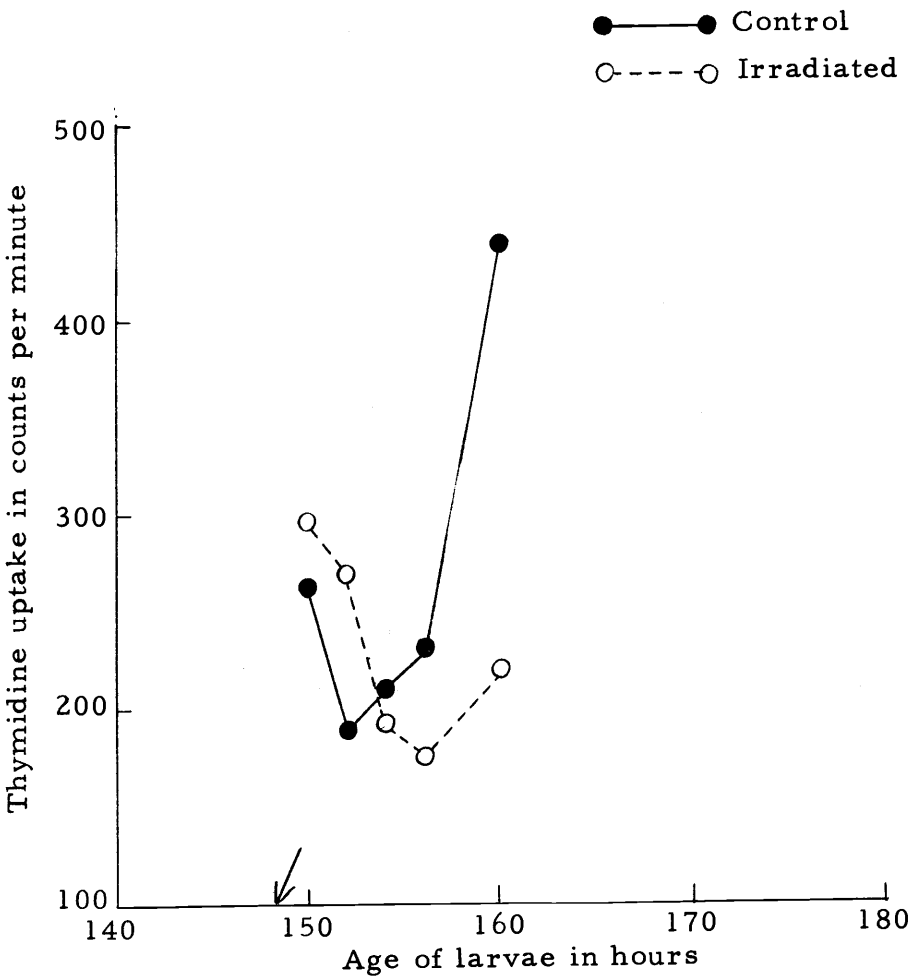


Figure 6. In vitro ^{3}H-thymidine uptake into salivary gland cells after 5000 R of whole body X-irradiation. Arrow indicates time of irradiation (148 hours). Thymidine uptake is measured in ten pooled glands and is expressed in counts per minute above background.

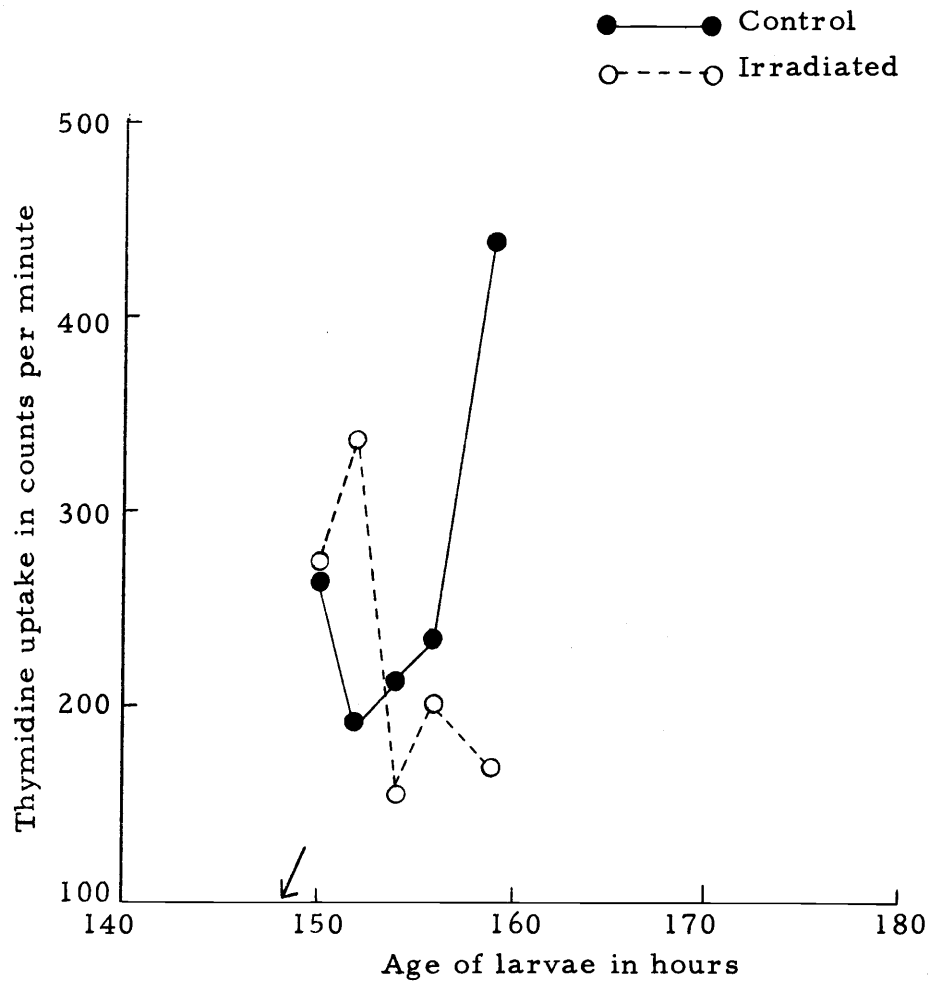


Figure 7. In vitro ^{3}H-thymidine uptake into salivary gland cells after 10,000 R of whole body X-irradiation. Arrow indicates time of irradiation (148 hours). Thymidine uptake is measured in ten pooled glands and is expressed in counts per minute above background.

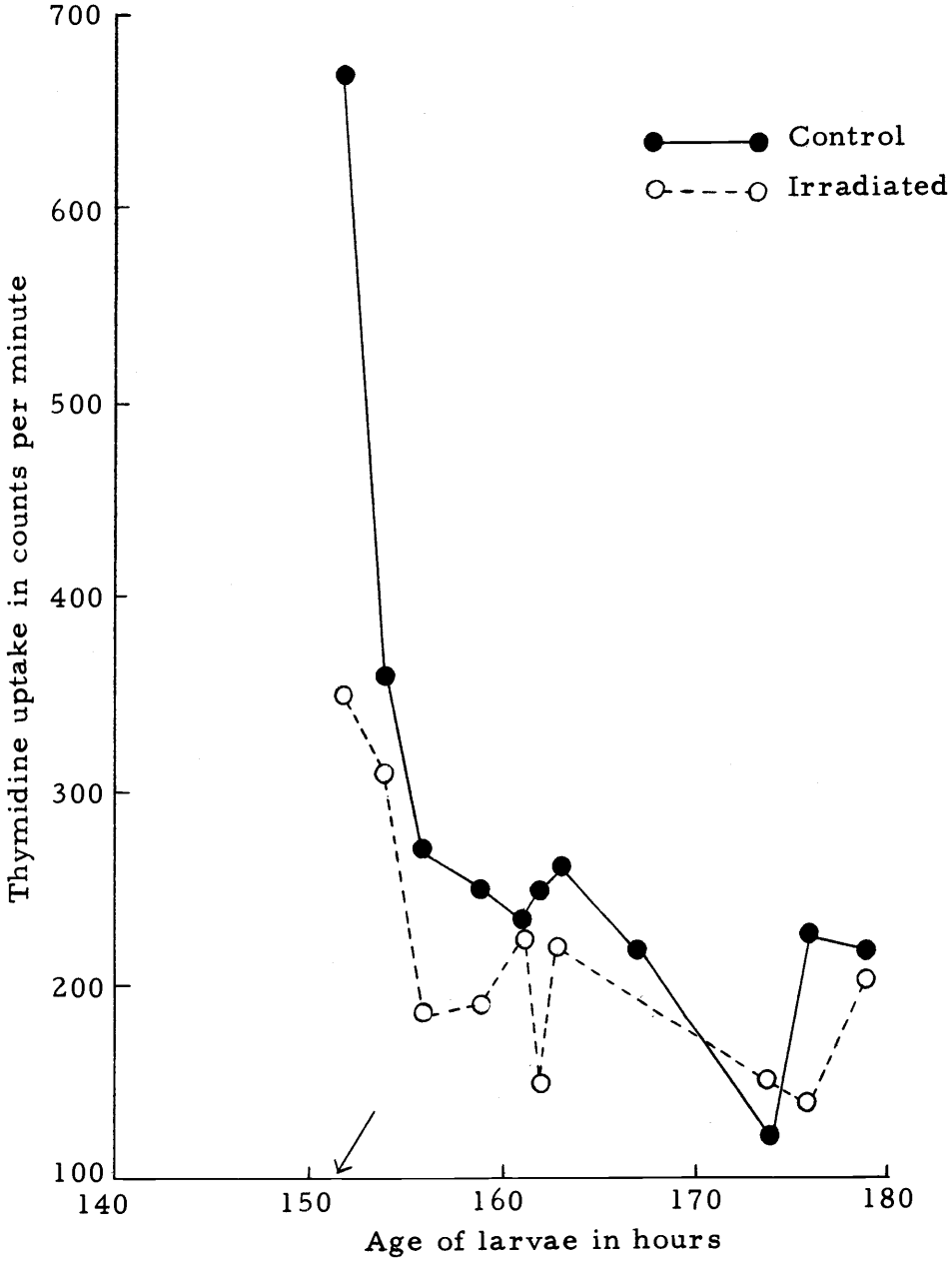


Figure 8. In vitro ^{3}H-thymidine uptake into salivary gland cells after 2500 R of whole body X-irradiation. Arrow indicates time of irradiation (151 hours). Thymidine uptake is measured in ten pooled glands and is expressed in counts per minute above background.

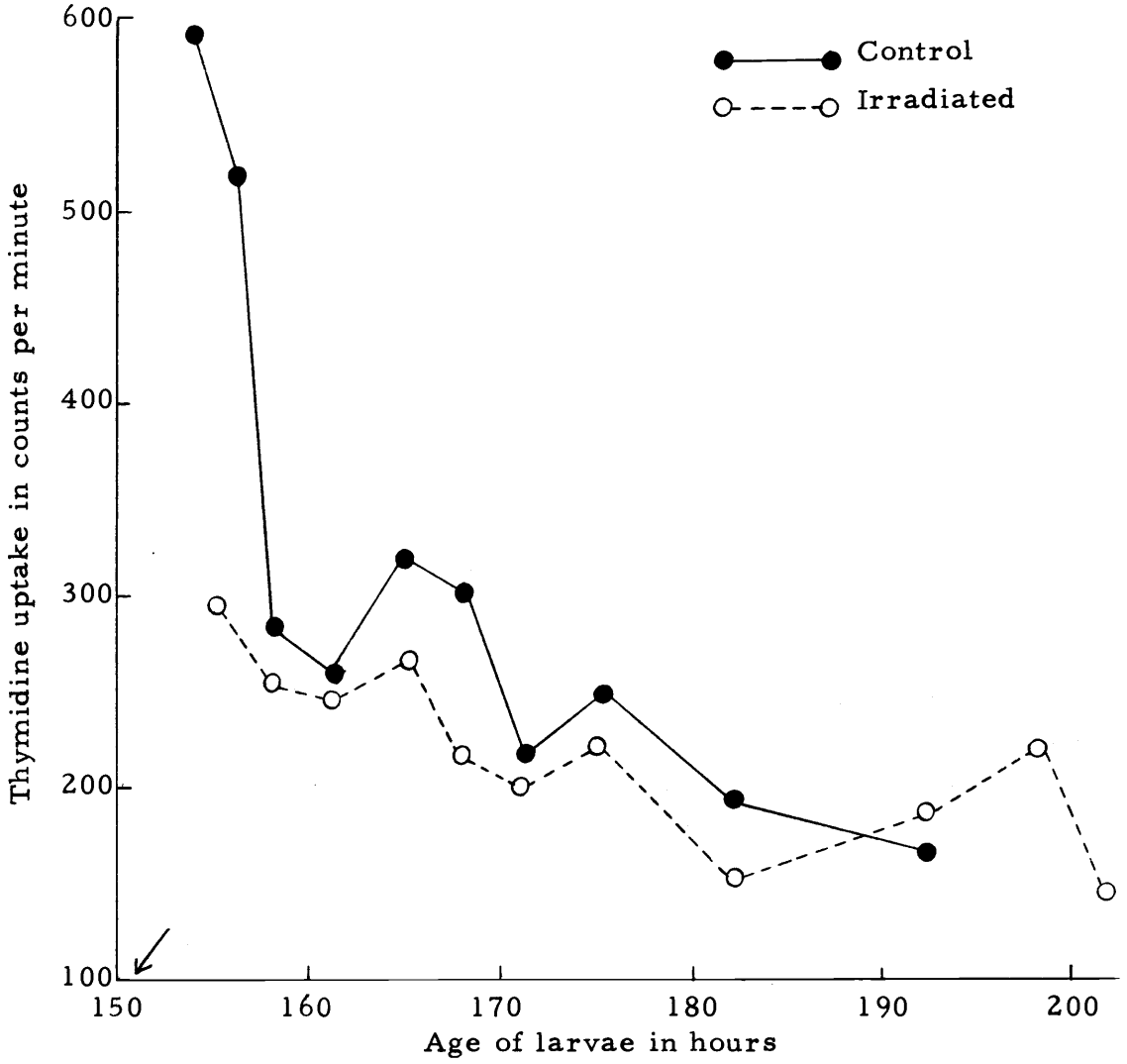


Figure 9. In vitro ^{3}H-thymidine uptake into salivary gland cells after 5000 R of whole body X-irradiation. Arrow indicates time of irradiation (151 hours). Thymidine uptake is measured in ten pooled glands and is expressed in counts per minute above background.

irradiated when between 144 and 151 hours of age, shortly before the onset of the MPS in the salivary gland (Figure 1). However, in two experiments (Figures 8 and 9), glands were already in the MPS when larvae were 151 hours old. Autoradiography was also used to study the short term effect of X-irradiation given to larvae before the MPS (149 hours old) and to larvae at the end of third instar development (200 hours old). The results are shown in Tables 1 and 2.

Short Term Effects in Early Third Instar Larvae

The short term effect of 1250-10,000 R of X-rays on DNA synthesis, when larvae were irradiated before the MPS, appears to be one of stimulation (Figures 2 through 7). Within three to seven hours after irradiation, measured ^{3}H-thymidine uptake is higher in the irradiated than in the control larvae. Although the stimulatory effect is slight and transitory, these results represent six cases of stimulation out of six trials. A sign test shows that this difference is significant at the 5% level. The stimulatory effect seems to be slightly higher after 1250 R than the other doses of X-irradiation. Otherwise, there is no apparent dose effect.

Autoradiography was used to determine if increased DNA synthesis after exposure of larvae to 1250 R of X-rays results from an increase in the number of cells synthesizing DNA or more DNA synthesis in each cell, or both. ^{3}H-thymidine uptake was measured in

Table 1. Effect of X-irradiation on thymidine incorporation in salivary gland cells of 149 hour old larvae.

Radiation dose	Mean percent and 95% confidence limits of nuclei showing:				Total number of nuclei analyzed
	No label	Light label	Medium label	Heavy label	
Control	62.2 ± 18.3	16.3 ± 7.4	15.5 ± 5.0	5.9 ± 3.8	373
1250 R	51.5 ± 15.9	13.8 ± 5.0	14.7 ± 5.2	20.0 ± 4.4	470

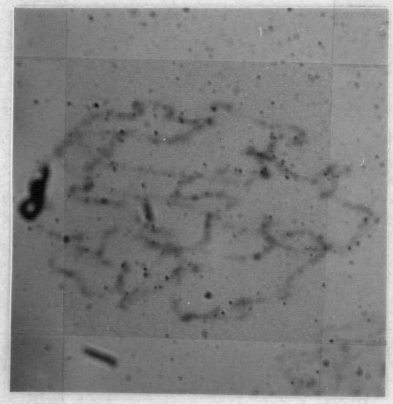
*From anterior half of each gland.

Table 2. Effect of X-irradiation on thymidine incorporation in salivary gland cells of 200 hour old larvae.

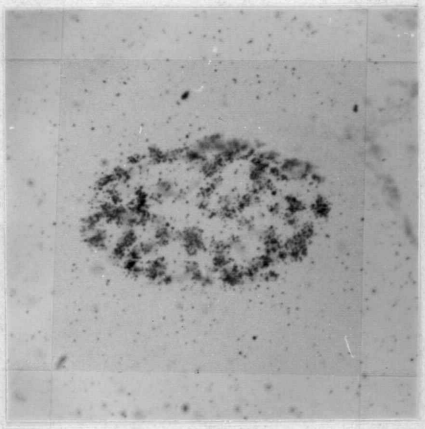
Radiation dose	Mean percent and 95% confidence limits of nuclei showing:				Total number of nuclei analyzed
	No label	Very light label	Light label	Medium label	
Control	76.3 ± 2.8	6.7 ± 4.4	15.6 ± 0.6	1.4 ± 1.2	578
1250 R	88.1 ± 9.8	9.1 ± 7.3	2.3 ± 2.0	0.5 ± 0.6	605
2500 R	75.1 ± 10.6	16.7 ± 7.5	4.4 ± 3.8	3.8 ± 3.2	611
5000 R	61.1 ± 11.8	34.3 ± 9.5	1.7 ± 3.4	1.7 ± 3.4	238
10,000 R	64.9 ± 7.0	30.9 ± 5.3	2.1 ± 1.6	0.7 ± 0.8	590

salivary glands of larvae irradiated at 149 hours of age, presumably before the onset of the MPS. Cells were pulse labeled within one hour after irradiation. The results are shown in Table 1. Cell nuclei are classified as having no label, light label, medium label, and heavy label. A key to this method of classification is shown in Figure 10. Since nuclei in the posterior half of each gland show little or no label, scoring was simplified by analyzing only nuclei in the anterior half of each gland. A significantly greater number of heavily labeled nuclei is observed in the irradiated than in the control glands (Table 1). These heavily labeled nuclei are located in the anterior quarter of the gland and are much less frequently observed in the same part of the control gland. There is no significant difference between the irradiated and the control nuclei showing light and medium label. These results indicate that stimulation of DNA synthesis after X-irradiation is due to an increase in the number of cells in active DNA synthesis. It cannot be determined from these results whether there is an increase in DNA synthesis in each cell after irradiation since counting of silver grains is impracticable and the degree of DNA labeling is only at best a rough approximation.

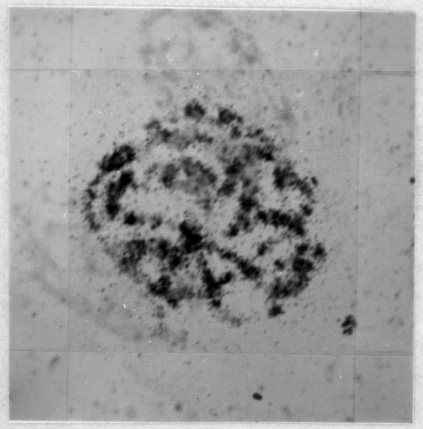
The short term effect of X-irradiation on DNA synthesis in two experiments is one of depression (Figures 8 and 9). In these experiments, larvae were unexpectedly exposed to 2500 and 5000 R of X-rays during instead of before the MPS in the salivary glands. This



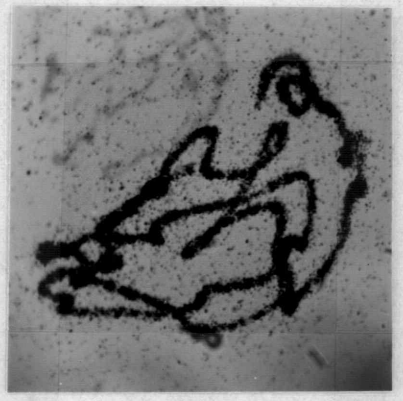
No label



Light label



Medium label



Heavy label

Figure 10. Autoradiographs of salivary gland cells from 149 hour old larvae showing various degrees of DNA label; incubated in ^{3}H-thymidine (25 μc/ml, about 11 Ci/mmole) for 1/2 hour, exposure time, four days. Feulgen stained.

can be explained by an increase in the overall rate of larval development due to unknown factors. When the duration of development in these larvae was estimated by timing pupation, it was found to be 10-15 hours shorter than expected. Thus the larvae must have been in a later stage of development compared to those used in other experiments at the time of irradiation. In these two cases, ^{3}H-thymidine uptake was markedly depressed within one to three hours after irradiation, indicating that the short term effect of X-irradiation, given during the MPS, is one of depression of DNA synthesis.

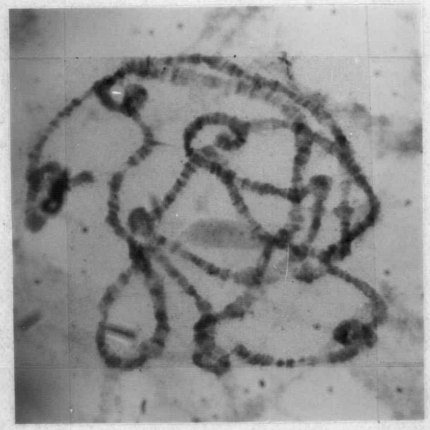
In conclusion, the present data show that the short term effect of X-irradiation on DNA synthesis in the salivary glands is one of stimulation or depression, depending on whether larvae were irradiated before or during the MPS. Stimulation may be accounted for by the induction of a group of cells in the anterior quarter of the gland into active DNA synthesis, as indicated by an increase in the number of heavily labeled nuclei in that part of the gland in the autoradiographic studies (Table 1).

Short Term Effects in Late Third Instar Larvae

Autoradiography was used to detect small amounts of unscheduled DNA synthesis as a possible sign of repair of X-ray damaged DNA in salivary gland cells. Larvae were exposed to 1250, 2500, 5000, and 10,000 R of X-rays at 200 hours of age. At this stage of

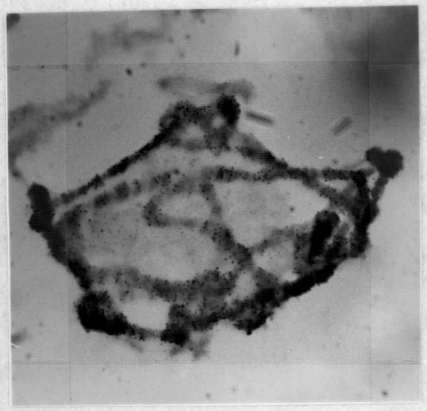
development, glands are usually filled with secretions and appear transparent. This stage lasts two to four hours, at the end of which histolysis occurs. Glands were pulse labeled within 30 minutes after irradiation. The results are shown in Table 2. Nuclei are classified as having no label, very light, light, and medium label as illustrated in Figure 11. In the present classification, no heavily labeled nuclei are included since they have not been observed; nuclei showing medium label have about the same grain density and nuclei showing light label are lower in grain density compared to those in Figure 10. On the average, nuclei classified as having very light label have anywhere from 50 to 200 grains above background. They are located at the more posterior part of the gland whereas nuclei classified as having light and medium label are located at the anterior end.

Larvae exposed to 1250 R of X-rays are lower than controls in the total number of labeled nuclei and in the number of lightly labeled nuclei but not significantly different in the number of nuclei showing very light and medium label; larvae exposed to 2500 R of X-rays are not significantly different in the total number of labeled nuclei and in the number of mediumly labeled nuclei. They are, however, significantly lower in the number of lightly labeled nuclei, and may or may not be significantly higher in the number of very lightly labeled nuclei. Larvae exposed to 5000 and 10,000 R of X-rays show very similar responses. They are significantly higher than control larvae

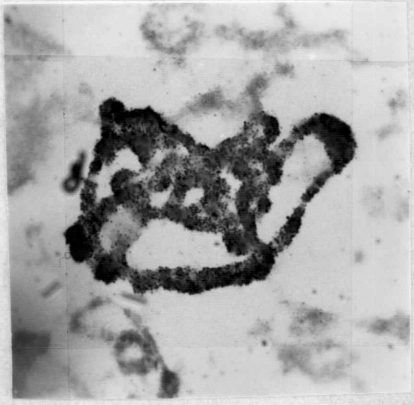


No label

Very light label



Light label



Medium label

Figure 11. Autoradiographs of salivary gland cells from 200 hour old larvae showing various degrees of DNA label; incubated in ^{3}H-thymidine (25 µc/ml, about 11 Ci/mmole) for 1/2 hour, exposure time, four days. Orcein stained.

in the total number of labeled nuclei and in the number of nuclei showing very light label, significantly lower in the number of lightly labeled nuclei and not significantly different in the number of nuclei showing medium label. The significant drop in the number of nuclei showing light label after all four doses of X-irradiation indicates immediate depression of DNA synthesis. The significant increase in the total number of labeled nuclei after 5000 and 10,000 R of X-irradiation indicates stimulation of DNA synthesis in cells that were in the non-synthetic phase (unscheduled DNA synthesis).

Long Term Effects

The long term effect of X-irradiation on DNA synthesis is predominantly one of depression. Within six to ten hours after 1250-10,000 R of X-irradiation, ^{3}H-thymidine uptake into salivary gland cells is depressed in larvae irradiated before the MPS (Figures 2 through 7). This depression occurs after the initial period of stimulation. As shown earlier, the depression effect is immediate when larvae were irradiated during the MPS in the salivary glands (Figure 8 and 9). The depression effect, once it comes into play, persists throughout the rest of larval development (Figures 3, 4, 5, 8, and 9. In all cases, the MPS is markedly suppressed. There is no apparent dose effect. These findings indicate that the predominant and overriding effect of X-irradiation on DNA synthesis in the

salivary gland cells is clearly one of depression.

Other Related Effects

The LD_{50} of early third instar larvae is approximately 3500 R as determined by the number of adult flies that emerged after irradiation (Wong, 1967b). Larvae exposed to 2500 and 5000 R develop to late pupal stages although both larval and pupal development are prolonged. This prolongation is directly proportional to the irradiation dose. The significance of some of these findings will be discussed later in this thesis.

DISCUSSION

DNA Synthesis in Salivary Glands

The data of the present studies indicate that there is a short period of intensive DNA synthesis (the MPS) in salivary glands of D. hydei just before the mid-point of third instar development (Figures 3, 4 and 5). This period occurs very abruptly and lasts, on the average, nine hours or roughly 5% of larval life. During this time, all or most of the DNA in the glands is probably being replicated at a fast rate. This is supported by the studies of Danieli and Rodino (1967) on DNA synthesis in the same species of Drosophila. Using the technique of autoradiography, these workers measured DNA synthesis in salivary glands at various stages of the third instar larvae and found a relatively short period (24 hours or roughtly 15% of larval life) of intensive DNA synthesis shortly before the mid-point of third instar development. This is in excellent agreement with the present data. Furthermore, during the period of intensive synthesis, these workers observed DNA labeling in 80% of the gland nuclei and measurement of DNA extracted from glands showed an increase in the contents of DNA amounting to at least two DNA doublings. These findings indicate that in the third instar larvae of D. hydei virtually all of the DNA in salivary glands is replicated very quickly at a specific stage in development.

DNA synthesis has also been studied in salivary glands of D. melanogaster (Rodman, 1967a). DNA synthesis at successive stages of the third instar larvae was measured by determining the DNA content of Feulgen stained nuclei using the technique of microspectrophotometry. The results indicate a gradual increase in DNA synthesis beginning in the early third instar larvae until three to five hours before the prepupal stage. At this time, a sudden wave of synthesis occurs within two to three hours and results, on the average, in two DNA doublings. These findings indicate that like D. hydei the DNA in salivary glands of D. melanogaster is also replicated very quickly at a specific stage in development; however, in this case, such replication occurs almost at the end instead of in the middle of third instar development.

It is clear from the present studies as well as the studies just described that in the metamorphosing larvae of Drosophila a regulatory mechanism is operative, determining the timing and rate of DNA synthesis in salivary glands. It has been suggested that DNA synthesis in insects is under hormonal control (Wigglesworth, 1964). In the lepidopteran Samia cynthia ricini, stimulation of DNA synthesis has been postulated as a primary effect of the hormone ecdysone (Krishnakumaran et al., 1965). However, it has been demonstrated in D. hydei that depression of DNA synthesis in salivary glands occurs when the level of ecdysone is high in the haemolymph (Danieli and

Rodino, 1967). Whatever the mechanism is for the regulation of DNA synthesis in the salivary gland, disturbances in this regulation would probably lead to changes in the timing as well as in the rate of DNA synthesis. Such considerations are crucial when studying the effect of X-irradiation on DNA synthesis in salivary glands since X-rays might disturb such a regulatory mechanism.

Stimulation of DNA Synthesis

In the present studies, exposure of larvae to X-rays before the MPS in salivary glands resulted in increased DNA synthesis shortly after irradiation. This increase is indicated by the data from liquid scintillation counting (Figures 2 through 7) as well as the data from autoradiographic studies in which more nuclei at the anterior part of the gland were observed to be heavily labeled after irradiation (Table 1, Figure 10). One way to explain this X-ray induced DNA synthesis is that it represents repair of damaged DNA. However, this explanation is not consistent with the autoradiographic studies just mentioned. If X-ray induced DNA labeling reflects repair of damaged DNA, one would expect to observe such DNA labeling in cells throughout the salivary gland instead of just in some cells at the anterior end. The present observation that DNA synthesis was depressed shortly after exposure of salivary glands to X-rays during the MPS (Figures 8 and 9) also argues against DNA repair; if repair of damaged DNA leads to

an increase in DNA synthesis, it would be hard to explain why no increase in synthesis was observed when glands were irradiated during the MPS. Alternatively, increased DNA synthesis after X-irradiation may be accounted for by precocious entry of cells into the MPS. This would explain why no stimulation of DNA synthesis was observed when glands were irradiated during the MPS since the majority of cells are already in the synthetic phase. Although it seems likely that X-ray induced DNA synthesis in salivary glands irradiated before the MPS reflects precocious synthesis, the possibility that some DNA repair is occurring cannot be ruled out. It may be that the amount of DNA labeling due to repair of damaged DNA is too small to be detectable in the presence of a great deal of DNA labeling resulting from semi-conservative replication.

The apparent induction of precocious DNA synthesis has also been demonstrated in HeLa cells using the technique of autoradiography (Feinendegen et al., 1968). An increase in DNA synthesis was observed after cells were exposed to moderate doses of X-irradiation (500 and 1000 R). Furthermore, this increase was found mostly in late G_1 cells. It was concluded that this X-ray induced DNA synthesis in HeLa cells results from a precocious entry of late G_1 cells into the normal S phase and/or repair. These findings, together with the present results on stimulation of DNA synthesis, indicate that precocious DNA synthesis might be a predominant effect of X-

irradiation in both insect and mammalian cells that are about to go into DNA synthesis.

Little information is available on the mechanism underlying radiation induced DNA synthesis of a semiconservative nature. The evidence so far seems to indicate that X-rays somehow trigger synthesis in cells close to the DNA synthetic phase in Drosophila as well as in HeLa cells. Kornberg (1969) has postulated that the initiation of normal DNA replication in Escherichia coli involves the introduction of a nick (equivalent to a single strand break) in the circular DNA molecule; the nicked region is bound to a site in DNA polymerase. It may be possible that X-irradiation mimics normal initiation of semiconservative replication in cells of higher organisms by the production of single strand breaks (many nicks ?) which may serve as starting points for DNA replication. However, this hypothesis is not consistent with the data of the present studies indicating depression of DNA synthesis when salivary glands were irradiated during the MPS (Figures 8 and 9). At this time, enzymes for DNA synthesis are presumably available in gland cells since most of them are in the synthetic phase. If X-ray induced single strand breaks in the DNA do serve as starting points for semiconservative replication, one would expect an increase in the number of sites in the chromosome synthesizing DNA. Therefore, salivary glands irradiated during the MPS should show an increase in DNA synthesis instead of a decrease

as was observed in the present studies. An alternative explanation for precocious DNA synthesis in salivary glands after whole body irradiation may be that X-rays cause disturbances in the regulatory mechanism governing DNA synthesis in larval development. As mentioned earlier, such disturbances might lead to changes in the timing of DNA synthesis. This explanation, however, cannot be applied to HeLa cells since they were irradiated in vitro.

In the present studies, X-irradiation of salivary glands at the end of larval development also resulted in stimulation of DNA synthesis, as measured by autoradiography. This stimulatory effect is substantiated by an increase in the total number of labeled nuclei as well as in the number of nuclei showing very light label (Table 2, Figure 11), indicating the induction of cells into light DNA synthesis. This X-ray induced synthesis may be a manifestation of DNA repair. Since such synthesis was observed only after exposure of salivary glands to the higher doses (5000 and 10,000 R), a large number of damaged sites in the DNA is apparently required before one can detect DNA labeling as a result of repair. This is consistent with the observation in cultured mammalian cells that massive doses of X-rays in the range of 10,000 to 100,000 R are required to demonstrate repair of damaged DNA (Evans and Norman, 1968; Hill, 1957, Painter, 1968; Painter and Cleaver, 1967). However, in the present state of knowledge, it is hard to justify extrapolations from

mammalian cells to salivary gland cells of Drosophila. The possibility remains that the X-ray induced DNA synthesis at the end of larval development is of a semiconservative nature. That cells at the end of larval development have the potential for more semiconservative replication is indicated by the observation in a tumorigenic strain of D. melanogaster (Rodman, 1967b) that extension in the larval period by failure of pupation resulted in an increase in the number of DNA replication cycles. Furthermore, chromosomes of larval salivary glands implanted in adult abdomens have been shown to achieve dimensions and DNA content far exceeding those of the normally developing prepupa (Hadorn, Gehring and Staub, 1963; Hadorn, Ruch and Staub, 1964; Berendes and Holt, 1965). Thus alterations in the development of salivary glands may lead to more semiconservative DNA replication.

The apparent repair of DNA damage has been reported in the third instar salivary glands of D. melanogaster after moderate to high doses of X-irradiation (Plaut and Valencia, 1968). Using the technique of autoradiography, an increase in the number of labeled nuclei as well as in the number of sites of DNA synthesis in the polytene chromosomes was observed when salivary glands were irradiated both invitro and as transplants in the abdomen of adults. Increased DNA synthesis is interpreted as an indication of DNA repair. The details of these experiments are not known. However, based on the

data of the present studies, it is entirely possible that the X-ray induced DNA synthesis observed in D. melanogaster is a manifestation of precocious DNA replication. In the absence of biochemical data such as those obtained in bacteria (Pettijohn and Hanawalt, 1963, 1964) and in cultured mammalian cells (Painter and Cleaver, 1969), in which the technique of equilibrium density centrifugation to detect repair replication was used, it would be very difficult to distinguish between DNA repair and precocious semiconservative replication.

The mechanism of DNA repair has not yet been studied in insects. It would be interesting to see if repair of damaged DNA in Drosophila can be explained by the "cut-and-patch" hypothesis. With its well known genetics, this organism may provide an excellent system for genetic studies on DNA repair as well as DNA synthesis in insects.

Depression of DNA Synthesis

The present data show that salivary glands irradiated before the MPS resulted in decreased DNA synthesis after an initial period of stimulation (Figures 2 through 7). This depressive effect was also observed very shortly after irradiation when glands were exposed to X-rays during the MPS (Figures 8 and 9). In both cases, depression of DNA synthesis was sustained throughout the rest of larval development after irradiation (Figures 3, 4, 5, 8, and 9). Decreased

synthesis was also observed in autoradiographic studies in which glands were irradiated at the end of larval development (Table 2); this is indicated by a decrease in the number of nuclei showing light label within 30 minutes after irradiation. These findings indicate that both the short term and long term effect of X-irradiation on DNA synthesis is one of depression.

The short term effect seems to suggest that the mechanism or mechanisms for depression of DNA synthesis by X-rays is operative immediately after irradiation. One such mechanism may involve changes in the thymidine pool as a result of DNA degradation by irradiation or changes in permeability of membranes (Adelstein and Manasek, 1967a, b). Inactivation of enzymes in the DNA-synthesizing system may also explain short term depression (Bacq and Alexander, 1961). However, doses much higher than the ones used in the present studies are usually required for such inactivation, especially when irradiation is given in vivo (Bollum et al., 1960). The effect of ionizing radiation on cellular processes in mammalian cells such as oxidative phosphorylation and formation of toxic substances described in the introduction of this thesis can also be used to explain the short term depression of DNA synthesis. In the absence of biochemical data in Drosophila, it would be difficult to attribute the depressive effect to any particular alteration or alterations in cellular processes after irradiation.

In addition to the changes in cellular processes mentioned above, the long term effect of depression of DNA synthesis in salivary glands may be explained by prolongation of larval development observed in the present studies. It is conceivable that the rate of DNA synthesis in the salivary gland is also slowed down. This is consistent with the present observation that extension of the larval period by X-irradiation also results in a prolongation of DNA synthesis (Figures 5 and 9).

Depression of DNA synthesis has not been reported previously in Drosophila or in other Dipteran. The present studies clearly show that the predominant effect on DNA synthesis in salivary glands of D. hydei is one of depression. More studies will be required to elucidate the nature of the mechanism underlying such effects.

SUMMARY

The effect of X-irradiation on DNA synthesis in larval salivary glands of Drosophila hydei was studied using the technique of liquid scintillation spectrometry and autoradiography. A major period of DNA synthesis, the MPS, was found in salivary glands before the mid-point of third instar development. Exposure of glands to X-rays before the MPS resulted in increased DNA synthesis shortly after irradiation; this increase was interpreted as precocious DNA synthesis. Exposure of glands to X-rays at the end of larval development also resulted in increased DNA synthesis but to a much lesser degree; this increase was interpreted as a possible sign of DNA repair although one cannot rule out the possibility that the increase is due to normal semi-conservative replication. The short term effect of X-irradiation on DNA synthesis when glands were irradiated during the MPS is one of depression. The long term effect when glands were irradiated before or during the MPS is also one of depression. Depression of DNA synthesis was explained by alterations in cellular processes observed in mammalian systems; in addition, the long term depressive effect was accounted for by an X-ray induced prolongation of the larval period, implying a slowing down in the rate of DNA synthesis. The mechanisms for precocious DNA synthesis and repair of DNA damaged are discussed.

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APPENDIX

Effect of X-irradiation on ^{3}H-thymidine uptake into salivary glands

Experiment number*	X-ray dose	Age of larvae (hours)	Counts per minute	
			Control	Irradiated
1	Non treated	140	300	-
		145	280	-
		147	308	-
		150	340	-
		152	322	-
		154	304	-
		155	530	-
		156	652	-
		158	728	-
		161	590	-
		164	367	-
		167	328	-
		170	350	-
		171	470	-
		174	227	-
		177	320	-
		181	390	-
		184	246	-
		186	240	-
		190	190	-
		192	180	-
		196	147	-
		200	200	-
		204	80	-
2	1250 R	149	353	423
		151	256	372
		153	230	432
		155	298	352
		158	408	195
		160	470	236
3	2500 R	154	324	341
		156	237	262
		158	231	135
		160	302	203
		162	605	180
		166	280	225
		168	250	224
		171	301	215
		182	225	200
		201	172	160

Experiment number*	X-ray dose	Age of larvae (hours)	Counts per minute	
			Control	Irradiated
4	2500 R	148	243	325
		150	270	240
		152	484	314
		154	295	225
		156	145	135
		158	220	155
		160	230	210
		162	255	190
		166	180	188
		171	284	186
		174	125	150
		176	225	205
		180	195	130
		188	234	140
5	5000 R	145	265	390
		147	226	345
		150	400	320
		152	-	360
		156	640	310
		158	620	400
		160	360	324
		162	464	330
		164	420	290
		168	295	240
		170	410	300
		178	360	240
		190	60	140
		194	-	150
		196	-	170
6	5000 R	150	264	297
		152	192	272
		154	211	194
		156	231	177
		160	440	220
7	10,000 R	150	264	275
		152	192	338
		154	211	155
		156	231	200
		159	440	170

Experiment number*	X-ray dose	Age of larvae (hours)	Counts per minute	
			Control	Irradiated
8	2500 R	152	668	350
		154	360	310
		156	270	185
		159	250	190
		161	230	226
		162	250	150
		163	260	220
		167	218	-
		174	122	150
		176	227	140
		179	219	202
9	5000 R	155	593	295
		156	521	-
		158	282	256
		161	261	251
		165	321	267
		168	305	220
		171	220	202
		175	250	224
		182	195	156
		192	173	187
		198	-	220
		202	-	150

*Corresponds to Figure number.