PHYSIOLOGICAL EFFECTS OF AN ALKYLATING AGENT, TEPA, ON THE DESERT LOCUST SCHISTOCERCA GREGARIA FORSKAL

by

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B. Sc., Simon Fraser University, 1968

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

June, 1970

APPROVAL

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Degree: Master of Science

Title of Thesis: Physiological effects of an alkylating agent, tepa, on the desert locust Schistocerca gregaria Forskal

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ABSTRACT

Although alkylating agents have been used to induce sterility in insects, little is known of their effects on the physiology and biochemistry of insects. The purpose of this investigation was to acquire a more comprehensive understanding of the effects of an alkylating agent tepa, tris(1-aziridinyl)phosphine oxide, on certain aspects of the physiology of reproduction in the female locust, Schistocerca gregaria.

Studies to determine the sensitivity of female Schistocerca gregaria and fifth instar nymphs to various concentrations of tepa showed that the fifth instar nymphs were more sensitive to tepa than the adults. A dose of 30 μg of tepa administered to adults reduced fecundity by approximately 50%, whereas in the fifth instar nymphs a dose of 26 μg was sufficient to bring about a 50% reduction in fecundity. Moreover this dose delayed the imaginal molt as well as maturation of the surviving adults. The reduction in fecundity was probably due to a high incidence of resorption of oocytes observed in the tepa-injected locusts. The effects of tepa were concentration dependent.

To determine whether treatment with tepa inhibited the synthesis of hemolymph proteins female fifth instar nymphs were injected with 26 μg of tepa. After the imaginal molt the hemolymph protein concentrations of tepa-injected and control insects were determined at 24 hour intervals over a 24-day

period. The hemolymph protein concentrations were significantly lower in the tepa-injected locusts than in those of the controls.

When the fat body from the treated and control groups was incubated in the presence of leucine-C^{14} it was observed that the specific activity of both fat body proteins and of proteins released by the fat body was significantly lower in the tepa-injected group than in the controls. It is concluded that the reduction in the hemolymph protein concentration observed in the tepa-injected group is due to inhibition in the synthesis of proteins as well as their release from the fat body.

Amounts of DNA in individual fat body nuclei of 1- and 6-day-old fifth instar female nymphs of S. gregaria were determined microspectrophotometrically on Feulgen-stained fat body nuclei. The results show that the fat body cells contain multiple DNA classes even in the 1-day-old nymph. Further polyploidization appears to take place during the growth of the nymph. The DNA classes do not correspond to a doubling series. Injection of 26 μg of tepa into nymphs during the mid-phase of their 5th stage has a slight inhibitory effect on DNA synthesis. Autoradiographic studies with thymidine-H^3 show that the intensity of labelling of nuclei in the tepa-injected insects is less than that of the controls. The most significant effect of tepa is a 40% increase in nuclear diameter.

Treatment with tepa reduces the colour intensity of

Feulgen-stained nuclei. This may be due to the fact that alkylation of DNA prevents the unmasking of aldehyde functions necessary for recoloration of Schiff's reagent.

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ACKNOWLEDGMENTS

I am indebted to Dr. K. K. Nair for his valuable assistance in the preparation and writing of this manuscript. My thanks go also to Drs. J. S. Barlow, C. L. Kemp and A. P. van Overbeeke for their suggestions and comments. I am indebted to W. F. Dean and G. Hollingdale for their technical assistance in this research.

GENERAL INTRODUCTION

One of the most promising approaches to pest control in recent years is that of induced sterility. Reducing or eliminating reproductive potential without otherwise affecting behaviour has been successful in controlling some insect pests (Borkovec, 1966). The best known example of this method was the eradication of screw-worm flies from Curacao and the south-eastern United States by the release of males rendered sexually sterile by ionizing radiation (Baumhover et al., 1955; Knipling, 1960). By overwhelming the natural population with sterile screw-worm flies for several generations, the fertile individuals in the natural population experience progressively greater odds in encountering fertile mates until the chances for successful fertile matings reach zero. However, when the density in a natural population is very high the cost of rearing and releasing large numbers of sterile insects becomes prohibitive (Lindquist, 1968).

The development of effective chemical substitutes for radiation has broadened the application of the induced sterility approach to pest control. Individuals of a natural population are exposed to these chemicals, called chemosterilants, in essentially the same manner that insects are exposed to insecticides for destruction (Borkovec, 1966). In addition chemosterilants are more economical and more practical than

gamma radiation because the rearing of large numbers of insects for release is not necessary.

In a very broad sense insect chemosterilants may be defined as chemicals which deprive insects of their ability to reproduce. This definition necessarily excludes compounds that either directly or indirectly interfere with or prevent mating and includes compounds that may only indirectly affect the gonads (Borkovec, 1966). Until recently the majority of chemicals effective as insect sterilants were cancer chemotherapeutic agents or closely related compounds (Borkovec, 1966). Chemosterilants are therefore classified according to a system used in cancer research. Five categories of chemicals are distinguished: alkylating agents, mitotic poisons, antimetabolites, radiomimetic compounds, and miscellaneous agents (Borkovec, 1962). Due to the classification system which is partly chemical and partly biological there are many compounds that fall into more than one category.

Chemosterilants may bring about sterility in insects in a number of ways: induction of dominant lethal mutations in either sex, failure to develop oocytes or sperm, destruction or inactivation of oocytes or sperm after they are produced (La Chance et al., 1968). To be useful as a chemosterilant chemicals must not seriously affect competitiveness or longevity, therefore somatic damage must be minimal and the

major effects must concentrate on germ tissues. Although a great number of chemicals are available for insect chemosterilization and many different insects have been sterilized (Knipling, 1968), much remains to be learned regarding the mode of action of these chemicals.

The category of chemical compounds appearing to offer the greatest promise of success as chemosterilants is the biological alkylating agents. A biological alkylating agent is a reagent which functions by accepting an electron pair from carbon in an organic reaction under physiological conditions (Turner, 1968). The polar compounds or nucleophiles with which they react donate the electron pair to the carbon in the reaction. Alkylation is, then, a substitution of an alkyl group for a hydrogen atom on a nucleophile with the whole process termed nucleophilic substitution. Various aliphatic, aromatic and heterocyclic analogues of ethylenimine, all of which are aziridinyl compounds, are the biological alkylating agents that chemosterilants research is generally focussed on. Other groups included as biological alkylating agents are methane sulfonates and mustards.

Sterility effects of alkylating agents are probably due to the alkylation of some target nucleophile preventing its utilization in the reproductive process. The ease with which these agents react with nucleophiles in living systems suggests that a number of reactions may occur simultaneously and that several

target molecules may be involved (Turner, 1968). The molecular basis for sterilization has not yet been established.

Sterility in female insects may result from either infecundity or by the production of inviable eggs. The most important factor governing which of these effects prevails is the timing of treatment (La Chance et al., 1968). Oogenesis is a sequence of events beginning with mitotic division of oogonial cells and terminating with the deposition of eggs. The process is controlled by genetic, hormonal and environmental factors. Up to a certain point in this process interference by alkylating agents results in infecundity characterized by a number of physiological effects. Interference with oogenesis after this stage results in sterility caused by damage to the genetic material in the oocyte nucleus and consequent production of inviable eggs (Borkovec, 1966). The importance of timing was demonstrated by Crystal and La Chance (1963) and La Chance and Leverich (1965) who studied the effects of alkylating agents on screw-worm flies. One group of flies contained nurse cells in which endomitosis was taking place while the other group of flies was older and endomitosis was complete. Inhibition of ovarian growth was much greater in the first group and resulted in almost complete infecundity. Treatment of the second group had little effect on fecundity but eggs laid did not hatch.

In female insects the most commonly observed effect of alkylating agents is inhibition of ovarian development resulting in reduced fecundity (Morgan and La Brecque, 1962, 1964; Rai, 1964; Hair and Adkins, 1964; Smittle, 1964; Schwartz, 1965; Keiser et al., 1965). A variety of these compounds produce changes which range from complete necrosis of the organ to only slight reduction in its size and the severity of the effect is undoubtedly concentration dependent.

A number of physiological effects have been observed at the cellular level in female insects. Treatment of screw-worm flies with alkylating agents before endomitosis is complete prevents nurse cells from reaching the degree of polyploidy necessary to sustain the synthetic activity required during oogenesis (La Chance and Leverich, 1965). Further, La Chance and Leverich (1968) found that second and third egg chambers failed to develop which indicated damage to germarium cells. In female mosquito larva (Aedes aegypti) the aziridinyl compound, apholate, caused degeneration of cells of the follicular epithelia with clumping of chromatin material (Rai, 1964). Damage to follicle cells and nurse cells was observed in ovaries of the house fly (Musca domestica) after treatment with a number of alkylating agents (Landa and Rezabova, 1965).

At the molecular level it has been shown that alkylating compounds such as aziridines can react with nucleic acid at

the N - 7 position of guanine (Brookes and Lawley, 1961). Kilgore and Painter (1964) observed a significant reduction in the levels of DNA and lactic dehydrogenase of eggs laid by house flies treated with the alkylating agent apholate. In a later communication, they (Painter and Kilgore, 1967) reported that another compound, thiotepa induced a similar reduction in the DNA content of eggs. Chamberlain and Barrett (1968) observed that treatment of stable flies (Stomoxys calcitrans) with apholate inhibited incorporation of tritiated thymidine into the DNA of nurse cells and follicular cells.

Apart from these studies little is known of the effects of alkylating agents on the physiology and biochemistry of insects. The purpose of this investigation was to acquire a more comprehensive understanding of the effects of the alkylating agent tepa, tris (1-aziridinyl) phosphene oxide, on certain aspects of the physiology of reproduction in the female desert locust, Schistocerca gregaria Forskal. The objectives of the study were: I. To determine the sensitivity of the fifth instar and female adult locusts to various concentrations of tepa, considering: (1) mortality, (2) molting (fifth instar), (3) maturation and (4) fecundity. II. To determine the effects of tepa on (1) concentration of hemolymph protein in adults of various age groups and (2) synthesis of proteins and their subsequent release from fat body cells. III. To deter-

mine the effects of tepa on DNA synthesis in nuclei of fat body cells.

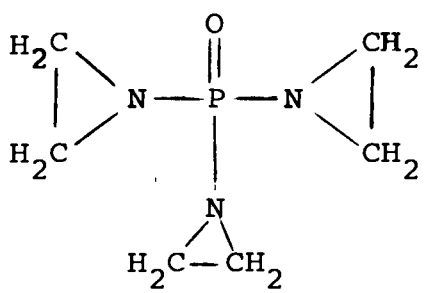
Chapter 1

I. SENSITIVITY OF FIFTH INSTAR NYMPHS AND FEMALE ADULTS TO VARIOUS CONCENTRATIONS OF TEPA.

INTRODUCTION

The literature on chemosterilization of insects is voluminous and has been adequately reviewed by Borkovec (1966) and LaBrecque and Smith (1968). These studies have shown the potentiality of a wide variety of compounds, including alkylating agents, as chemosterilants in a wide spectrum of insect species.

Tepa, tris (1 - aziridinyl) phosphine oxide, is one of the typical aziridinyl compounds investigated as a chemosterilant. It is also known by the following synonyms: TEP, APD, aphoxide, triethylene phosphoramide, SK3818, NSC9717 and ENT2415. Turner (1968) reviewed the chemical and physical properties of tepa. It is a colorless, odorless crystalline solid melting at 41° C and boiling at 90 - 91° C. It is known to undergo violent decomposition if heated above 130° C, is very hygroscopic and unstable in aqueous solutions. Tepa is also very soluble in water, alcohol, ether and acetone. The structural formula of this compound is:



$C_6H_{12}N_3OP$ Mol. Wt. 173.16

Tepa has been successfully used in the sterilization of the following insects: Aedes aegypti, Anopheles quadrimaculatus (Weidhaas et al., 1961) Musca domestica (LaBrecque, 1961; Gouck, 1964) Stomaxys calcitrans (Harris, 1962) Blattella germanica (Burden and Smittle, 1963) Cochliomyia hominivorax (Crystal, 1963) Panonychus citri (Cressman, 1963) Culex pipiens quinquefasciatus (Mulla, 1964) Musca autumnalis (Hair and Adkins, 1964) Ceratitis capitata, Dacus cucurbitae, Dacus dorsalis (Keiser et al., 1965) Anastrepha ludens (Shaw and Riviello, 1965) Prionoxystus robinae (Solomon, 1966) Diabrotica balteata (Creighton et al., 1966) Fannia canicularis (Davis and Eddy, 1966) Ostrinia nubilalis (Harding, 1967) Hyper postica (McLaughlin and Simpson, 1968) Popillia japonica (Ladd, 1968).

The above studies showed that tepa can induce sterility in adults whether administered along with the diet or by topical application. Even contact of an insect with this compound has been shown to induce sterility, as has been demonstrated in the mosquito, Aedes aegypti (Weidhaas, 1962). Although the sensitivity to tepa of several species of holometabolous insects has been investigated little is known of the sensitivity of hemimetabolous insects to this compound. Paucity of information on the sensitivity of S. gregaria to tepa necessitated investigations to determine the minimum quantity of tepa which brings about a significant reduction in ovary development with

little or no mortality in the adults. These investigations are presented in this chapter.

MATERIAL AND METHODS

The locusts used were initially obtained from a laboratory colony maintained at the University of British Columbia. The insects were reared under constant light at a temperature of 33 - 36° C, a relative humidity of 60 - 70% and on a diet that consisted of a mixture of whole wheat bran and fresh grass. The bran mixture was made up as follows:

6 parts whole wheat bran

2 parts cara grass meal

1 part white brewer's yeast

1 part powdered skim milk

Various concentrations of tepa were made from a stock solution containing 80% tepa in methanol. Except during use all solutions containing tepa were refrigerated. The solutions were injected with a Hamilton constant rate microsyringe calibrated to deliver 5 μl. Control locusts were injected with 5 μl of methanol. Adult females as well as fifth instar females were injected with various concentrations of tepa to give from 5 to 50 μg per locust (Table I, II). Twenty five to eighty insects were used at each concentration of tepa.

Prior to injection, the locusts were anesthetized with carbon dioxide. Injections were given by inserting the needle 2 - 3 mm into the hemocoel through the arthrodial membrane at

the base of the second pair of legs. After injection, the female locusts were placed in rearing cages with untreated mature males. Mortality was recorded at 24 hour intervals for 21 days. A female was considered sexually mature when it received a male for mating. The number of days required for a female to show this response after the imaginal molt is referred to as the maturation time. Fecundity was assessed on the basis of the number of eggs per pod laid during the first gonadotrophic cyle, which took place between the 16th and 24th day after adult emergence.

RESULTS

Effects of tepa on adults

There was no mortality in the control group and the number of eggs per pod was 70 ± 3.7 (Table I). Injection of 5 or 10 μg of tepa per locust did not affect the survival or the maturation time. Moreover, there was no significant effect on the number of eggs laid per pod ($P > 0.05$). Although a dose of 20 μg of tepa per locust caused 21% mortality within 21 days, there was no difference in the maturation time of these treated locusts. But, the number of eggs laid per pod was significantly reduced to 50 ± 3.1 ($P < 0.01$). Injection of 30 μg of tepa caused 49% mortality and the maturation time was increased by 5 days. The number of eggs laid per pod was significantly reduced to 40 ± 2.1 ($P < 0.01$). Injections of 40 and 50 μg of tepa resulted in 81 and 100% mortality respectively. No mating was observed in surviving locusts injected with these amounts of tepa, nor did they lay eggs.

Effects of tepa on fifth instar nymphs

Table II summarizes the effects of four concentrations of tepa injected into fifth instar nymphs. The duration of the fifth instar was 6 - 7 days in the control group. A dose of 22 μg of tepa did not delay the imaginal molt nor was mortality noted in the fifth instar, but 22% of these died after the

imaginal molt. The number of eggs laid per pod by the surviving adults was significantly reduced to 51 ± 3.5 ($P < 0.01$). Doses of 26, 29 and 32 μg delayed the imaginal molt by 3 days. Mortality both in the fifth instar and the adult stages increased with increasing doses. Also, with increasing doses of tepa the number of eggs per pod decreased significantly ($P < 0.01$) and at a dose of 32 μg no eggs were deposited.

Mating in the control group was observed 14 days after adult emergence. Injection of 22, 26 and 29 μg of tepa per insect increased this time to 15, 19 and 22 days respectively. No mating was observed in those injected with 32 μg of tepa.

Egg viability

Although treatment with tepa reduced the number of eggs deposited, it did not appear to affect the viability of these eggs. In the control and the tepa-injected groups of both experiments an estimated 70% of the eggs deposited hatched. Moreover, the incubation period of the eggs obtained from treated insects was the same as that of eggs laid by the control group. This period was found to be approximately 14 days in both groups.

Gross morphology of ovaries

In S. gregaria the paired panoistic ovaries lie in the abdomen, one on either side of the mid line. Each ovary is made

up of approximately 50 ovarioles, the proximal ends of which are thread-like, whereas the distal ends thicken and become tubular in structure. The tubular portion is divided into a number of follicles which contain the developing oocytes. The most developed oocyte, the terminal one, is nearest the oviduct.

Although some morphological changes were observed in the ovaries of tepa-injected adults, only ovaries of adults injected with tepa as fifth instar nymphs were studied in detail. A number of adults that metamorphosed from tepa-injected fifth instar nymphs had asymmetric ovaries, one being smaller than the other. The degree of this effect increased with increasing doses of tepa.

Examination of the ovaries in the groups treated with doses of 26 μg or more showed the presence of orange-red bodies in some of the terminal oocytes. These have been described by Lusis (1963) as resorption bodies and are formed when the contents of a developing oocyte are resorbed leaving behind the orange-red pigment, β-carotene. Various stages of resorption (RB1 to RB6) as described by Lusis (1963) were observed in the ovaries of insects treated with 26 μg of tepa and the total number of oocytes thus affected ranged from 13 to 27 in a pair of ovaries. Resorption of oocytes was not observed in any of the ovaries of control females.

TABLE I

Effects of various concentrations of tepa on adult S. gregaria.

Tepa µg/insect	Number of Females	Cumulative Mortality (%)			Maturation Time	Eggs/Pod First Cycle
		7 Days	14 Days	21 Days		
0	25	0	0	0	15	70±3.7
5	25	0	0	4	14	71±3.8
10	25	0	0	0	15	69±4.9
20	25	4	12	21	16	51±3.1
30	25	8	21	49	20	40±2.1
40	25	8	40	81	--	0
50	26	60	100	100	--	0

TABLE II

Effect of various concentrations of tepa on fifth instar nymphs of S. gregaria.

Tepa µg/insect	Number of Females	% Mortality in Fifth Instar	Days in Fifth Instar	% Mortality in Adults (14 Days)	Maturation Time	Eggs/Pod First Cycle
0	64	0	6- 7	0	14	68±4.2
22	80	0	6- 7	22	15	51±3.5
26	80	12	9-10	24	19	37±2.6
29	80	19	9-10	53	22	30±4.4
32	48	37	9-10	75	--	0

DISCUSSION

It is evident from the results that the fifth instar nymphs are more sensitive to tepa than adults with respect to mortality as well as fecundity. Treatment in the nymphal stage delayed and in many cases, depending on the concentration, prevented adult emergence. Sharma and Rai (1969) similarly observed that another alkylating agent, apholate, can interfere with growth and metamorphosis of Aedes aegypti larvae.

In hemimetabolous insects the physiological events leading to metamorphosis are probably similar. The nymphal epidermal cells of Rhodnius prolixus exhibit intense mitotic activity prior to each molt. This is brought about by the action of the growth hormone, ecdysone, on the epidermal cells (Wigglesworth, 1933; 1954). Baldwin and Salthouse (1961) showed that irradiation of Rhodnius nymphs resulted in a delay in mitosis in the epidermal cells with a corresponding delay in molting. It is well known that alkylating agents can induce genetic disturbances in cells which, in turn, result in an appreciable delay in mitosis (Loveless, 1966). Therefore it is reasonable to assume that one of the factors responsible for the delay in molting observed in the tepa-injected S. gregaria fifth instar nymphs is caused by a delay or inhibition of mitosis in the epidermal cells.

Treatment with tepa induced the resorption of 13 - 27% of the terminal oocytes leading to the formation of resorption bodies. Although female S. gregaria that have gone through a number of gonadotrophic cycles showed varying numbers of resorption bodies in the ovaries, they were not found in untreated females during the first gonadotrophic cycle. A variety of factors appear to affect oocyte resorption in S. gregaria (Highnam and Lusis, 1962; Lusis, 1963; Highnam et al., 1963; Highnam, 1964). These include non availability of protein (hemolymph proteins), impairment in the neurosecretory activity and/or low titre of juvenile hormone. Whether treatment with tepa is accompanied by all of the above is not known. Subsequent studies showed a significant reduction in hemolymph proteins of tepa treated locusts (Chapter 2). Hence the increase in the resorption bodies found in tepa treated locusts is probably due to the lower levels of hemolymph proteins.

The objective of this study was to determine the minimum quantity of tepa that would bring about a significant reduction in ovary development with little mortality in the adults. A dose of 26 μg injected into fifth instar nymphs caused a 50% reduction in fecundity. Although higher doses reduced the fecundity further the mortality was high in both fifth instar and adult stages. Hence a dose of 26 μg of tepa has been chosen for subsequent studies reported in Chapters 2 and 3.

Chapter 2

II. EFFECT OF TEPA ON HEMOLYMPH PROTEIN CONCENTRATION AND PROTEIN SYNTHESIS IN THE FAT BODY.

INTRODUCTION

Wigglesworth (1943) first suggested that hemolymph proteins could be taken up directly by the developing oocytes when he observed that hemoglobin derivatives were found in the oocytes of a blood-sucking insect, Rhodnius prolixus. Telfer (1954), using immunochemical techniques, provided conclusive evidence for this when he demonstrated the preferential accumulation of sex-specific proteins from hemolymph by the oocytes in Hyalophora cecropia.

Hill (1962) observed cyclic changes in the concentration of hemolymph proteins in female S. gregaria during oocyte development. The concentration of hemolymph proteins increased during the early period of yolk deposition. This was followed by a decrease when yolk deposition was complete. The decrease in protein concentration was due to the uptake of proteins by the oocytes since in ovariectomized S. gregaria the concentration of hemolymph proteins continued to rise. Orr (1964) observed a similar phenomenon in Phormia regina.

Although the storage function of the insect fat body is well known it has become evident that considerable metabolic activity also takes place in this organ. In regard to protein synthesis various transaminases present indicate that the fat body is a site for active amino acid metabolism (Kilby, 1963). The fat body is also involved in fat and carbohydrate metabolism

and in certain detoxification reactions (Kilby, 1963). Several workers have considered the fat body to be the important site of hemolymph protein synthesis (Shigematsu, 1958; Price, 1966, 1967; Price and Bosman, 1966; Chippendale and Kilby, 1969). Hence any changes in the protein synthetic activity of the fat body may be reflected in the hemolymph protein concentration.

In the previous chapter it was suggested that the increase in the incidence of resorption of oocytes followed by a reduction in fecundity in tepa-injected locusts was due to a lowering of hemolymph protein concentration. Therefore the following study was made to determine whether there was a change in the concentration of hemolymph proteins in tepa-injected locusts and whether such a change was accompanied by changes in the synthesis and release of proteins from fat body cells.

MATERIALS AND METHODS

Since it was found that 26 μg tepa injected into fifth-instar nymphs of S. gregaria induced approximately 50% reduction in fecundity (Chapter 1), the same dose was used to study its effects on hemolymph protein concentration, protein synthesis in the fat body and protein release into the hemolymph. Four-day-old fifth-instar female nymphs were injected with 26 μg of tepa.

Hemolymph proteins

Hemolymph protein determinations made began 12 and 24 hours after the imaginal molt, thereafter daily for 24 days. Hemolymph samples were obtained by severing the dorsal vessel in the last abdominal segment and allowing hemolymph to flow onto clean parafilm squares. Disposable micropipets were then used to obtain two 5 μl samples from each locust. Five locusts were used at each time to give 10 hemolymph samples. A total of 250 locusts were used in this study.

Each hemolymph sample was added to 2 ml of 5% trichloroacetic acid (TCA) to precipitate the protein. After centrifugation the precipitate was washed in 5% TCA, centrifuged and the precipitate washed twice in 3:1 ethanol:ether. The total protein in each sample was estimated according to the method of Lowry et al. (1951). Known concentrations of bovine serum

albumin were used as standards. Optical density was determined at 500 nanometers in a Unicam SP500 spectrophotometer.

After obtaining the hemolymph samples the locusts were dissected and the ovaries examined to determine the time at which yolk deposition in the terminal oocytes became apparent.

Protein synthesis in the fat body

The most significant difference in the hemolymph protein concentration in the control and tepa-injected groups occurred on the 9th day after the imaginal molt. Therefore, fat body from 9-day-old locusts was used for the analysis of the effect of tepa on protein synthesis. For the incubation of fat body tissue a medium containing all essential amino acids except leucine was used (medium A, Stephenson and Wyatt, 1962; see Appendix 1). Osborne et al. (1968) found that this medium is suitable for incubation of locust fat body.

Locusts were anesthetized with CO_2 and subsequently their fat body was dissected out in the above medium at approximately 4° C. The perivisceral fat body was removed as a sheet, freed from air sacs and tracheae and blotted on filter paper. Each fat body was placed in a weighed 10 ml flask containing 0.9 ml of the incubation medium and 0.1 ml of a solution of 1 μCi of leucine-C^{14} (specific activity 311 m Ci/mM). Flasks were kept on ice until the dissection and weighing of the individual

tissue samples of all locusts used in the experiment had been completed. Approximately 100 mg of fat body was used for each incubation. Flasks were placed in a metabolic shaking incubator at 30° C for 15, 30 or 60 min. Twelve samples were used for each incubation period (6 from controls and 6 from tepa-injected locusts).

At the end of each incubation period the reaction was stopped by placing the flasks in an ice bath. The fat body was removed from the incubation medium, rinsed in 1 ml of the medium and transferred to 4 ml of 70% ethanol. This was then placed in a water bath (100° C) for 5 minutes. After cooling it was homogenized and transferred to centrifuge tubes. After centrifugation the fat was removed from the sample by sequentially extracting with 100% ethanol (twice), and ethanol:ether, 3:1 (twice), or until no radioactivity was detected in the supernatant. After draining the tubes, 2 ml of 1N NaOH were added to each sample and the tubes were placed in a water bath (100° C) for 5 minutes. From this solution 1.0 ml was used to estimate the amount of protein (Lowry et al., 1951) and 0.1 ml was placed in 12 ml of scintillant for measurement of C^{14} activity. The scintillant was made according to Bray (1960) except the ethylene glycol monomethyl ether was substituted for ethylene glycol. The C^{14}-activity of each sample was then measured over a 10 minute period or until a preset statistical counting error

of ±1% was reached on a Beckman-250 liquid scintillation system. The counting efficiency was 60%. All counts were corrected for background activity and the specific activity of the proteins is expressed as counts per minute (CPM) per mg of protein.

Protein released by the fat body

Upon termination of the incubation period, each medium was treated with 1 ml of 10% TCA (twice) to precipitate the proteins. This precipitate was then washed three times in ethanol:ether (3:1) and subsequently the amount of protein as well as its C^{14}-activity was determined as described above.

The data obtained in the above investigations were analyzed statistically using the t-test.

RESULTS

Hemolymph proteins

The hemolymph protein concentration in the control and tepa-injected groups are presented in Table III and Figure 1. Statistical analysis of the daily differences between the two groups showed that they were significant ($P < 0.01$) with the exception of those of day 1, 2, 6, 19 and 20.

Twelve hours after the imaginal molt hemolymph protein concentration was approximately 4g/100 ml in both control and tepa-injected groups. By day 5 it had decreased to 1.6g/100 ml in the control group, whereas in the tepa-injected group the lowest value (1.5g/100 ml) was not reached until day 9. After the initial decline, the protein concentration rose to a maximum of 6.8g/100 ml in the control and 5.6g/100 ml in the days after adult emergence, whereas in the tepa-injected group the maximum was reached 19 days after adult emergence. This was followed by a decline in both groups.

Protein synthesis in the fat body

Incorporation of leucine-C^{14} into fat body proteins increased with increase in incubation time in control as well as tepa-injected locusts. However, in the latter the rate of incorporation was significantly lower than that in the former group (Fig. 2, Table IV).

Protein released by the fat body

The amount of protein released from the fat body into the incubation medium also increased with increase in incubation time. But in the tepa-injected locusts the amounts were lower than those in the controls (Table V).

The specific activity of the released protein showed an increase in both groups with respect to time. But only after one hour of incubation was it significantly higher in the control than in the tepa-injected group (Fig. 3; Table VI).

TABLE III

Hemolymph protein concentrations in control and tepa-injected adult female S. gregaria.

Age of locust in days	Hemolymph protein concentration g/100 ml $\bar{x}$ ±S.D.		Comparison of means
	Control	Tepa-injected	
0.5	3.9±0.4	4.1±0.3	p>0.01
1	3.6±0.2	3.9±0.3	p>0.01
2	3.2±0.3	3.5±0.4	p>0.01
3	2.2±0.3	3.1±0.2	p<0.01
4	2.4±0.2	3.5±0.3	p<0.01
5	1.6±0.5	3.2±0.3	p<0.01
6	3.1±0.4	3.2±0.2	p>0.01
7	3.6±0.4	3.0±0.4	p<0.01
8	4.2±0.2	2.4±0.4	p<0.01
9	4.3±0.3	1.5±0.5	p<0.01
10	4.1±0.3	1.8±0.3	p<0.01
11	4.8±0.4	2.4±0.3	p<0.01
12	5.2±0.3	2.9±0.4	p<0.01
13	5.6±0.2	3.3±0.5	p<0.01
14	6.1±0.4	3.8±0.5	p<0.01
15	6.5±0.2	4.2±0.5	p<0.01
16	6.8±0.3	4.4±0.2	p<0.01
17	6.3±0.6	4.6±0.5	p<0.01

TABLE III (cont.)

Age of locust in days	Hemolymph protein concentration g/100 ml $\bar{x}$ ±S.D.		Comparison of means
	Control	Tepa-injected	
18	6.1±0.5	5.2±0.3	$p<0.01$
19	5.4±0.4	5.6±0.3	$p>0.01$
20	5.1±0.3	5.3±0.3	$p>0.01$
21	4.6±0.4	5.5±0.3	$p<0.01$
22	4.0±0.2	5.2±0.3	$p<0.01$
23	3.7±0.3	4.9±0.3	$p<0.01$
24	3.5±0.3	4.4±0.3	$p<0.01$

Figure 1. Effect of tepa on hemolymph protein concentrations in female S. gregaria. Locusts were injected as four-day-old fifth instar nymphs. Each point represents the mean ± standard deviation of 10 hemolymph samples, from five locusts. ●——● controls, ○——○ tepa-injected.

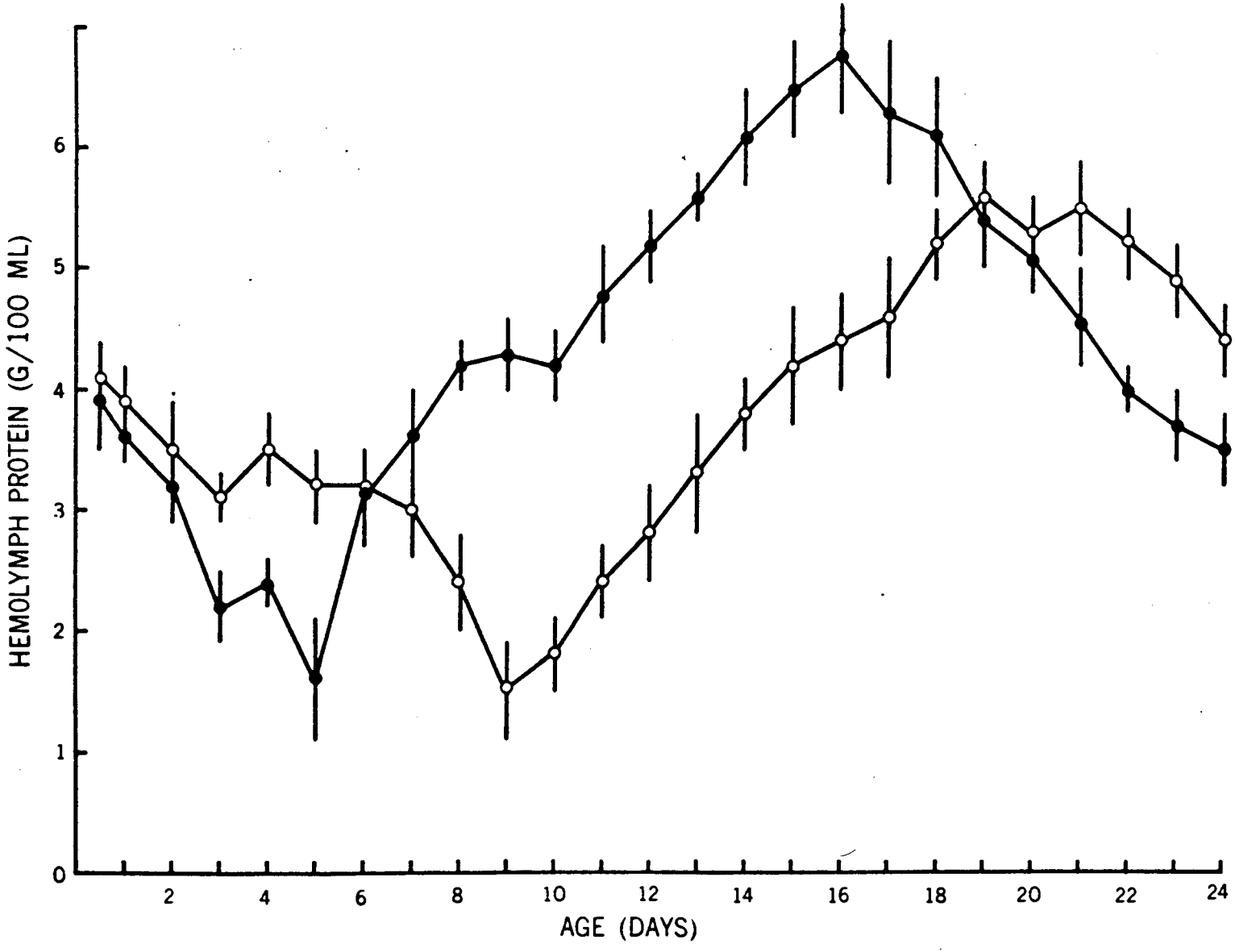

HEMOLYMPH PROTEIN (G/100 ML)
0
1
2
3
4
5
6
2
4
6
8
10
12
14
16
18
20
22
24
AGE (DAYS)

Figure 2. The effect of tepa on the incorporation of leucine-C^{14} into fat body proteins of female S. gregaria. Fat body was taken from 9-day-old adults that had been injected as 4-day-old fifth instar nymphs. Each point represents the mean ±S.E. of six fat body samples from six locusts. ●——● controls; o——o tepa-injected.

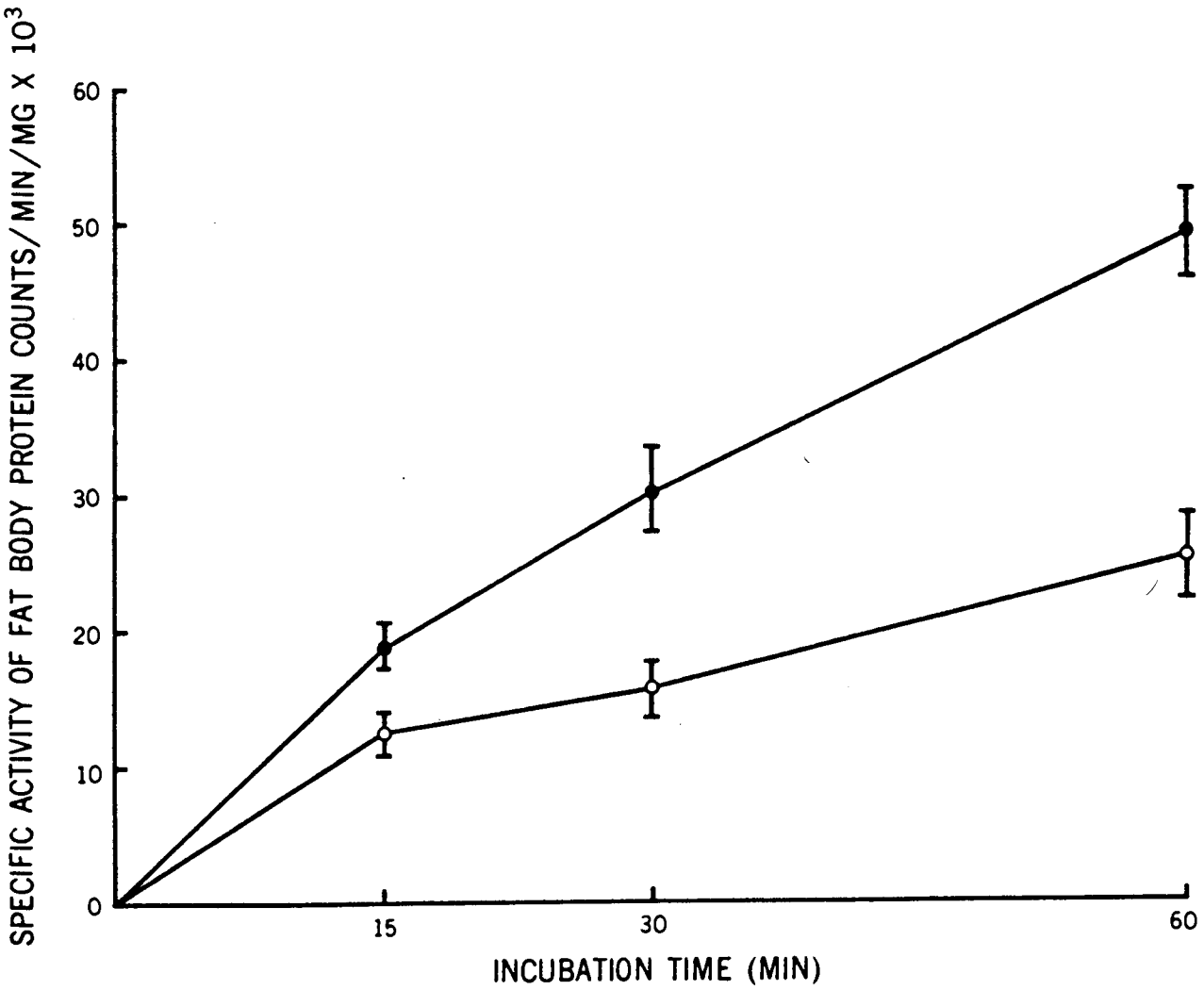

SPECIFIC ACTIVITY OF FAT BODY PROTEIN COUNTS/MIN/MG X 10^3
60
50
40
30
20
10
0
15
30
60
INCUBATION TIME (MIN)

TABLE IV

Effect of tepa on the incorporation of leucine-C^{14} into fat body proteins of female S. gregaria

Incubation Time (Min)	Specific Activity of Proteins x 10^3		Comparison of Means
	Control ($\bar{x}$±S.E.)	Treated ($\bar{x}$±S.E.)	
15	18.9±1.6	12.7±1.8	$p < 0.01$
30	30.3±3.0	15.6±2.1	$p < 0.01$
60	49.3±3.0	25.6±3.2	$p < 0.01$

TABLE V

Amount of protein released into the incubation media by fat body from control and tepa-injected female S. gregaria

Incubation Time (Min)	Released Protein as % of Fat Body Protein		Comparison of Means
	Control ($\bar{x}$±S.E.)	Treated ($\bar{x}$ ±S.E.)	
15	7.5±0.6	4.1±0.7	$p < 0.01$
30	10.2±0.9	6.7±0.6	$p < 0.01$
60	12.3±0.7	8.5±0.5	$p < 0.01$

Figure 3. The effect of tepa on the incorporation of leucine-C^{14} into proteins released from the fat body of female S. gregaria. The fat body used for incubation was from 9-day-old locusts injected with tepa as 4-day-old fifth instar nymphs. Each point represents the mean ±S.E. of the specific activity of proteins released by fat body from six samples.

●——● controls; o——o tepa injected.

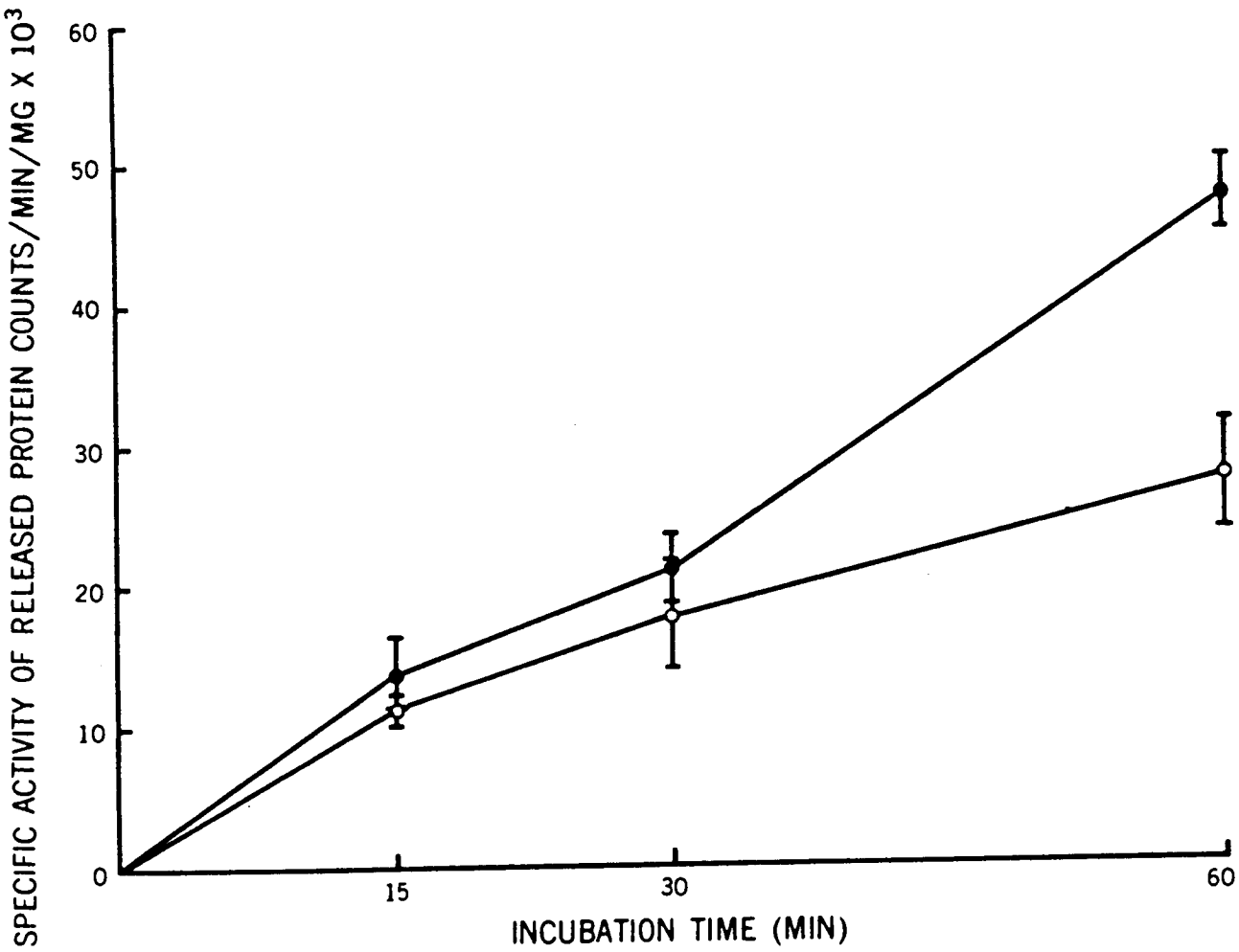

SPECIFIC ACTIVITY OF RELEASED PROTEIN COUNTS/MIN/MG X 10^3
60
50
40
30
20
10
0
15
30
60
INCUBATION TIME (MIN)

TABLE VI

Effect of tepa on the incorporation of leucine-C^{14} into proteins released from fat body of female S. gregaria

Incubation Time (Min)	Specific Activity of Proteins x 10^3		Comparison of means
	Control ($\bar{x}$±S.E.)	Treated ($\bar{x}$±S.E.)	
15	13.7±2.5	11.4±1.1	$p > 0.01$
30	21.1±2.4	17.8±3.7	$p > 0.01$
60	47.8±2.3	27.6±3.8	$p < 0.01$

DISCUSSION

Hill (1962) observed in S. gregaria that following imaginal molt, the hemolymph protein concentration increased from 1g/100 ml to 6g/100 ml within 10 days. He associated this increase with oocyte maturation, a process which is thought to be controlled by the neuroendocrine system. Hill, furthermore, suggested that the concentration of hemolymph proteins must reach approximately 4g/100 ml for yolk deposition to begin. The data obtained in the present study are generally in agreement with those of Hill. Yolk deposition was not apparent until the 10th day after the imaginal molt. At that time the hemolymph protein concentration was 4.1g/100 ml. That this concentration is indeed related to the onset of yolk deposition is further illustrated by the fact that in the tepa-injected locusts yolk deposition did not commence until day 14 when the protein concentration had reached 3.8g/100 ml.

However, in the present study it was observed that the concentration of hemolymph proteins is high initially and it declines rapidly to a low level of 1.6g/100 ml by day 5. Hudson (1966) also observed a drop in concentration of hemolymph proteins in fifth-instar larva of Protoparce quinquemaculata following ecdysis. This initial decrease in concentration of hemolymph proteins has been associated with a period of rapid growth of tissues. Bucher and his associates (Bucher, 1966)

observed in the migratory locust, Locusta migratoria, that differentiation of flight muscles begins immediately after the imaginal molt. Further they showed that the mitochondria increase in number and size. They found the rate of incorporation of C^{14}-labelled isoleucine into mitochondrial protein was highest on day 1 and had declined to a low value by day 5 when differentiation of flight muscles was complete. Although similar studies have not been carried out in S. gregaria, it is reasonable to assume that such changes are indeed taking place in its flight muscle. This is supported by the observation that the weight of the flight muscle in S. gregaria is more than doubled by the fifth day after the imaginal molt (Weis-Fogh, 1952). Hence the decline in concentration of hemolymph proteins after the imaginal molt can be associated partly with the differentiation of the flight muscles.

The decline in hemolymph proteins can also be due to sequestration of hemolymph proteins by fat body cells. That hemolymph proteins do enter fat body tissue has been shown in Rothchildia orizaba and Malacosoma americanum (Loughton and West, 1965), P. quinquemaculata (Hudson, 1966) and Calliphora erythrocephala (Price, 1967). Locke and Collins (1968) deduced from electron micrographs that fat body cells of Calpodes ethlius sequester hemolymph proteins. They suggested that protein is first concentrated in the intracellular spaces and channels by

loss of water. If a similar phenomenon is taking place in the fat body cells of S. gregaria it could then account for the rapid decline in the concentration of hemolymph proteins after the imaginal molt.

Although changes in hemolymph protein concentration in the tepa-injected locusts parallel those in the control group, the maximum concentration is significantly lower than that in the controls. As already stated, resorption of oocytes and consequent reduction in fecundity in tepa-injected locusts may be due to insufficient quantity of proteins in the hemolymph. The lower concentration of hemolymph proteins in tepa-injected locusts confirms the validity of the above assumption. It appears as though there is sufficient concentration of proteins to allow oocytes to begin development but not enough to allow all of these to complete it. One of the other factors that is responsible for resorption of oocytes in S. gregaria is lack of corpus allatum hormone (Highnam et al., 1963). In the present study no attempt was made to determine whether injection of tepa inhibited the synthesis and/or release of the corpus allatum hormone.

Functionally the fat body of insects is analogous to the vertebrate liver as it is considered to be the chief site of intermediary metabolism (Kilby, 1963). Using an in vitro system with Bombyx tissues Shigematsu (1958) established that

hemolymph proteins are synthesized by the fat body and then released into the hemolymph. Hill (1965) showed that the concentration of the C^{14}-labelled protein in the fat body of S. gregaria reached a maximum in one hour after injection of glycine-C^{14}, whereas specific activity of the hemolymph proteins showed a maximum only after four hours, which implies that hemolymph proteins are synthesized in fat body and then released. Price (1966) provided further evidence regarding the role of fat body vis-à-vis hemolymph protein synthesis. He found that when fat body of C. erythrocephala larva was incubated in the presence of valine-C^{14} the specific activity of released protein increased rapidly.

It was mentioned earlier that the reduction in fecundity in the tepa-injected locusts was probably due to lower concentration of hemolymph proteins. In the light of evidence presented regarding the role of fat body in hemolymph protein synthesis, the reduction in hemolymph protein concentration observed in the tepa-injected locusts is due to an inhibition in protein synthesis and its release from the fat body.

Chapter 3

III. POLYPLOIDIZATION IN THE FAT BODY CELLS OF FIFTH INSTAR NYMPHS OF *S. GREGARIA*

INTRODUCTION

Early attempts at the quantitative estimation of deoxyribonucleic acid (DNA) using biochemical methods (Boivin et al., 1948; Vendrely and Vendrely, 1948, 1949; Mirsky and Ris, 1949) resulted in the DNA constancy theory. However, these methods failed to demonstrate exceptions to the theory. Gross analyses of nuclear suspensions where only average DNA values of large numbers of nuclei are possible could not show discrepancies in the amount of DNA between single nuclei. More specifically, the polyploidy known to exist in some animal tissues (Swift, 1950) could not be detected.

Caspersson's pioneering work in 1936 brought about the development of ultraviolet microspectrophotometry which made possible the quantitative determination of DNA in single nuclei. The first photometric estimates of the amount of DNA per nucleus were made by Caspersson (1939) on the testes nuclei of the grasshopper Gomphocerus. Leuchtenberger and Schrader (1952) were the first to demonstrate polyploidy in isolated rat liver nuclei by this method.

In early studies difficulty in distinguishing DNA from RNA in nuclear absorption hampered photometric estimations of the amount of DNA per nucleus. Employing the specificity of the Feulgen reaction for DNA combined with microspectrophotometric

techniques has further advanced research on the quantitative estimation of the amount of DNA in single nucleus. Using this method Mirsky and Ris (1949) first reported the presence of multiple DNA classes in mammalian liver. Three nuclear DNA classes were found in a 1:2:4 ratio with the lowest class corresponding to the diploid value. Similar results have been obtained in other mammalian liver tissue using the same or a slightly modified method (Swift, 1950; Alfert, 1950; Geschwind et al., 1958). Clinical cytologists have also used these methods to demonstrate polyploidy and the corresponding increase in DNA levels in the diagnosis of malignant tumors.

In the previous chapter it was reported that treatment with tepa inhibited protein synthesis in the fat body of Schistocerca gregaria, but the mechanism involved in this inhibition remains to be established. Alkylating agents such as aziridines are known to react with DNA and proteins. That alkylating agents inhibit DNA synthesis in the ovaries of insects has been demonstrated at least in two species. Treatment of screw-worm flies with an alkylating agent before endomitosis in the nurse cells is complete prevents them from reaching the degree of polyploidy necessary to sustain the synthetic activity required during oogenesis (LaChance and Leverich, 1965). By autoradiographic techniques Chamberlain and Barret (1968) have demonstrated that treatment of young

stable flies (Stomoxys calcitrans) with apholate results in a considerable inhibition in the incorporation of thymidine-H^3 into DNA of nuclei of the nurse cells and follicle cells. This can lead to impairment of cellular functions. Although alkylating agents have been used for sterilization of a wide spectrum of insects little is known of their effects on cells other than those of gonads. Hence the present study was undertaken with a view to 1) obtaining information on polypoloidization in the fat body cells of fifth instar nymphs of S. gregaria and 2) determining the effect of tepa on this process.

MATERIALS AND METHODS

Microspectrophotometry

Squash preparations of fat body tissue from five 1-day-old and five 6-day-old fifth instar nymphs of S. gregaria were fixed in 10% buffered neutral formalin for 24 hours. They were washed overnight in running water and stained by the Feulgen technique as outlined by Deitch (1966). The optimum time of hydrolysis for maximum intensity of staining was found to be 10 minutes at 60° C in 1 N HCl (Fig. 4). The DNA content of 100 nuclei from each age group, relative to the amount of Feulgen stain, was determined on a Shimadsu MPS-50-W scanning microspectrophotometer. A quartz diffusion plate was used to minimize the "edge effect". All scans were made with a 50X objective and the diameter of the scanning aperture was 5 μ. Absorption spectrum of the Feulgen stained nuclei showed that maximum absorption occurred at approximately 560 nm (Fig. 5). The optical density obtained from the scan was multiplied by the diameter of the nucleus in microns to arrive at the DNA content in arbitrary units (AU). Using the above methods squash preparations of the testes from fifth instar nymphs were employed to obtain the DNA content in AU of 20 spermatogonial nuclei. These values were averaged to calculate the mean diploid value. One half of this represents the haploid

value (C). By dividing the value in AU for each fat body nucleus measured by C, a series of values was obtained, which subsequently was grouped into classes. The frequency distribution thus obtained is taken to reflect the polyploidy of the cells of the fat body.

To determine the effect of tepa on polyploidization, squash preparations fat body tissue were made simultaneously from five 6-day-old fifth instar nymphs that had been injected with 26 μg of tepa as four-day-old fifth instar nymphs. During these investigations I observed that although treatment with tepa did not change the absorption spectrum of the Feulgen-stained nuclei it interfered with the Feulgen reaction resulting in some loss in the intensity of staining (Fig. 6). (Details regarding this aspect are discussed in the next chapter). Therefore a correction factor had to be applied for the calculations of the DNA classes in the tepa-treated locusts. The C value was determined from 20 spermatogonial nuclei of tepa-injected (26 μg) fifth instar males. The DNA classes in the fat body nuclei were then calculated by dividing the individual AU by C.

Autoradiography

Autoradiographic techniques were used primarily to confirm the validity of some of the observations on polyploidization made with photometric techniques. They were not intended for a

detailed analysis of this process in the fat body of the fifth instar nymphs. This in itself could form the subject matter of further investigation.

Four-day-old fifth instar female nymphs were injected with 26 μg of tepa in 5 μl of methanol. Controls received 5 μl of methanol. Four hours later 20 μCi of thymidine-H^3 (specific activity 19.7 Ci/mM) into each of the control and tepa treated nymphs in two 10 μl samples four hours apart. Squashes of fat body of control and tepa injected groups (six insects were used from each group) were made when the nymphs were six days old. They were fixed in 10% neutral formalin for 24 hours. After thorough washing the slides were placed in cold 5% TCA containing 0.1% non-radioactive thymidine for 10 minutes. The slides were then washed in distilled water and stained by the Feulgen technique. The slides were coated with Kodak emulsion NTB-2 and stored at 4° C for 2 weeks. After developing and fixing the autoradiographs were examined for the percentage of labelled nuclei in both the groups. In addition the labelled nuclei were examined for the intensity of labelling and further classified into moderately labelled (Fig. 10) and heavily labelled nuclei (Fig.11, Table VIII).

Figure 4. Colour intensity of Feulgen-stained spermatogonial nuclei of S. gregaria as a function of hydrolysis time in 1N HCl at 60° C.

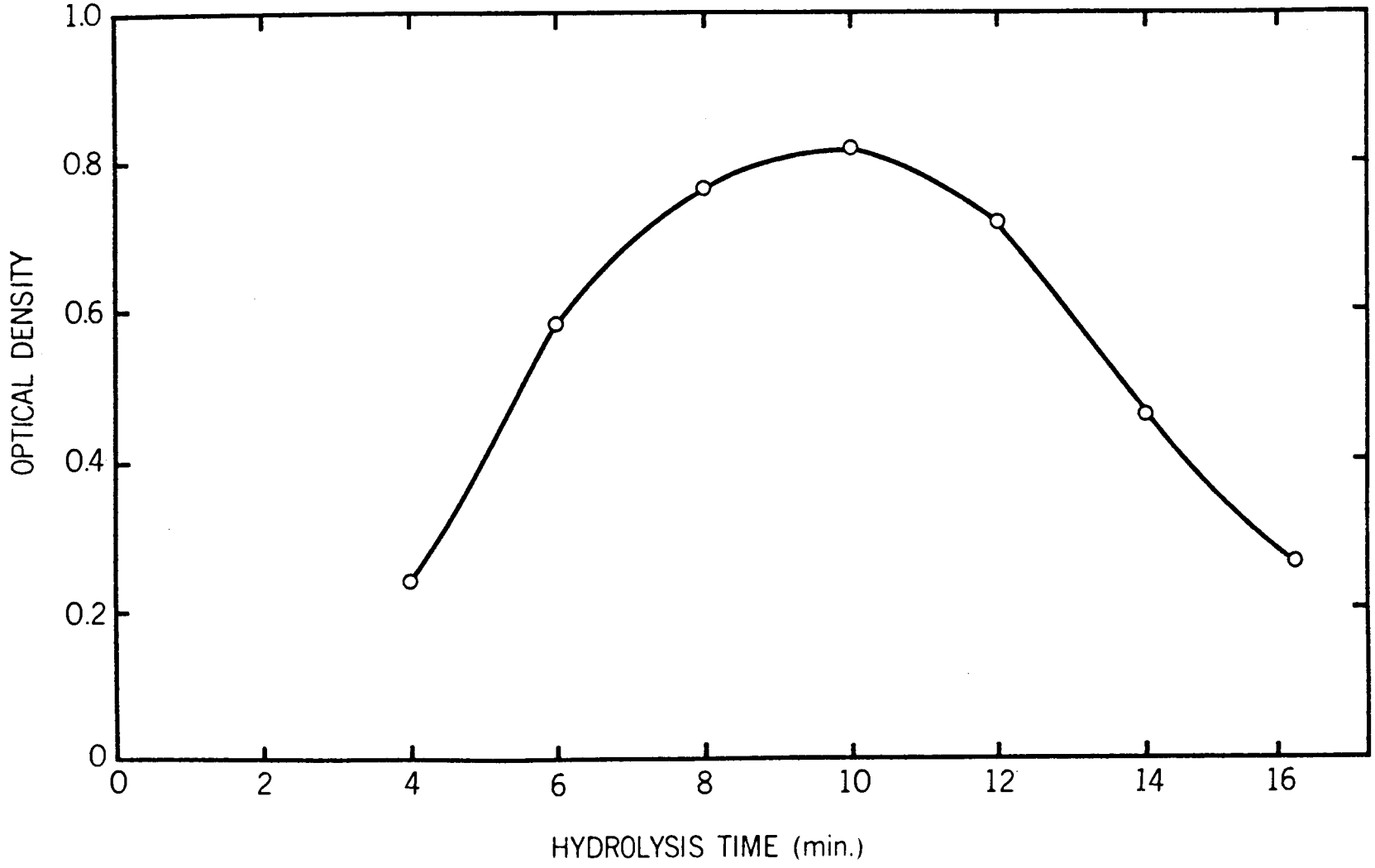

1.0
0.8
0.6
0.4
0.2
0
OPTICAL DENSITY
0
2
4
6
8
10
12
14
16
HYDROLYSIS TIME (min.)

Figure 5. Absorption spectrum (450 to 650 nm) of a Feulgen-stained fat body nucleus of fifth instar nymph S. gregaria.

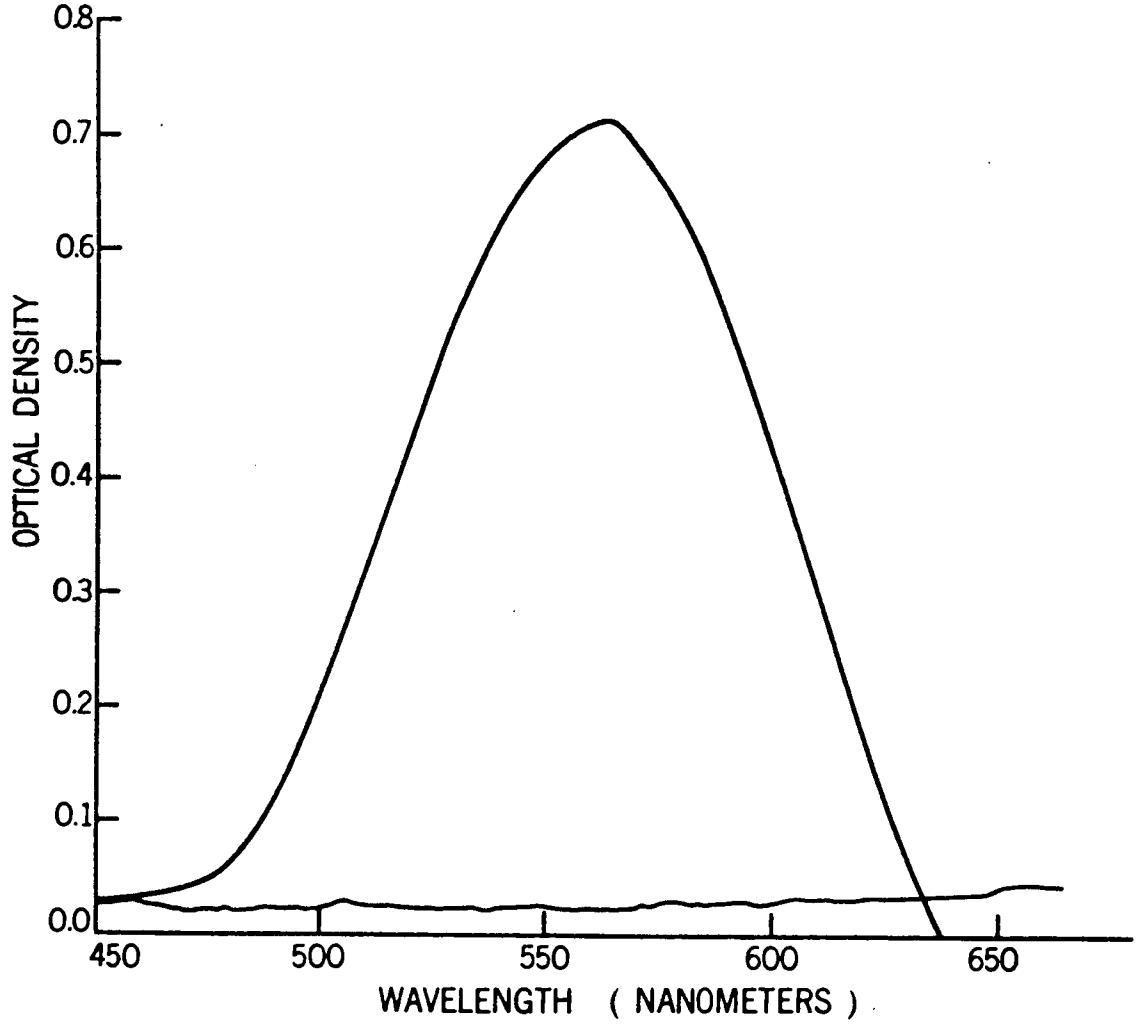
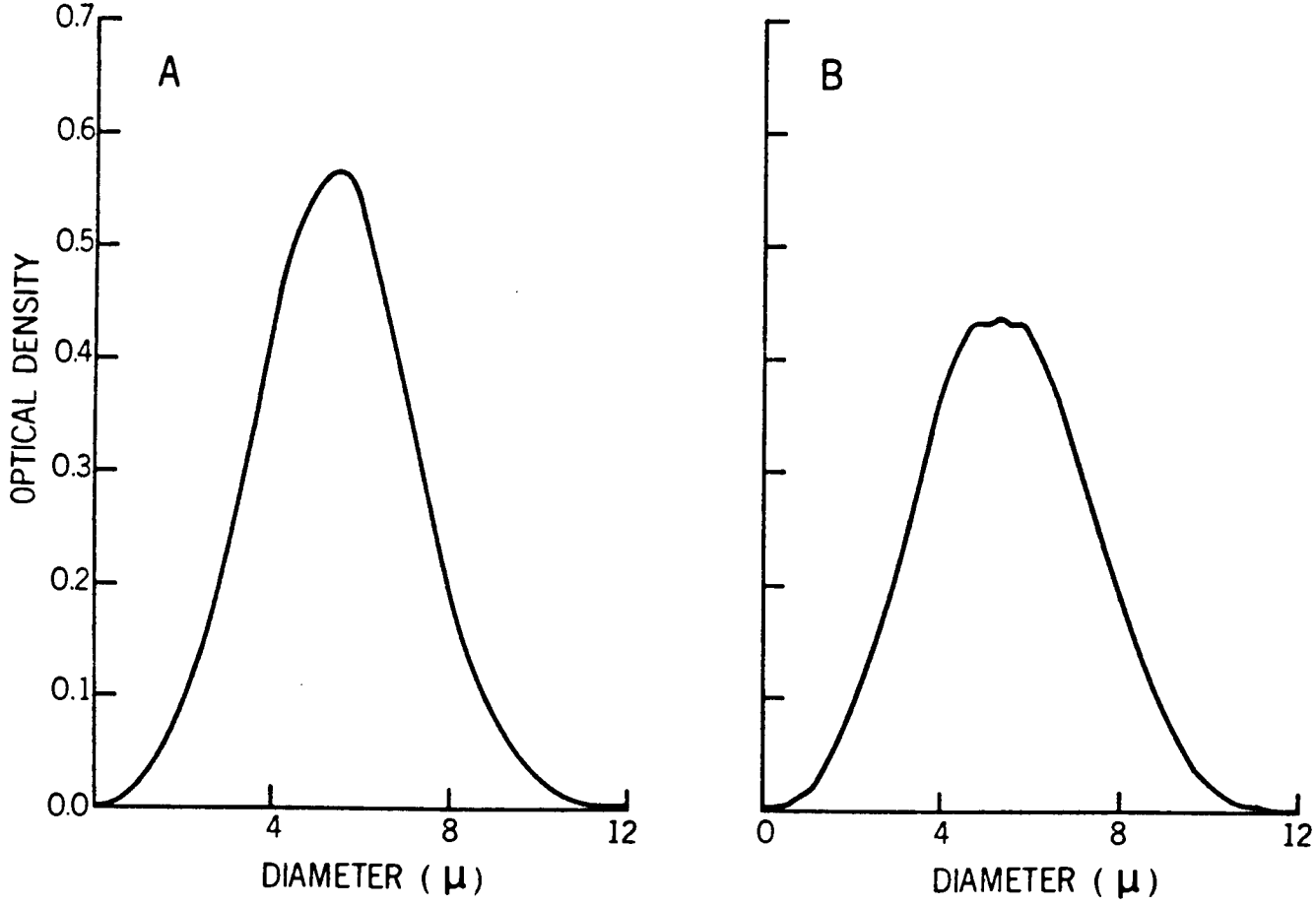

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OPTICAL DENSITY
450
500
550
600
650
WAVELENGTH (NANOMETERS)

Figure 6. Typical scan across Feulgen-stained spermatogonial nucleus in a control (A) and tepa-injected (B) fifth instar male S. gregaria. Optical density was measured at 560 nm. Note the reduction in intensity of staining in B.


A
B
OPTICAL DENSITY
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
4
8
12
DIAMETER (μ)
DIAMETER (μ)

RESULTS

Microspectrophotometry

The DNA content of the fat body nuclei of 1-day-old fifth instar nymphs ranged from 8 to 13 AU, whereas that of the 6-day-old nymphs ranged from 9 to 15 AU (Fig. 7A, B). When these values were divided by the haploid DNA value the DNA class-series obtained in the 1-day-old group obtained was: 3C (23%), 4C (54%) and 5C (23%). In the 6-day-old controls this series was: 3C (2%), 4C (60%), 5C (35%) and 6C (3%) of the nuclei (Figs. 8A, B; Table VII).

In the tepa-injected group the AU ranged from 7 to 13. Of these nuclei 64% were found between 7 and 10 AU (Fig. 7C). The C values obtained after making the necessary correction due to loss in Feulgen staining as a result of alkylation of DNA by tepa, showed that 14, 49, 26 and 11% of the nuclei were in 3C, 4C, 5C and 6C respectively (Fig. 8C; Table VII).

Analysis of the data on the nuclear diameter showed that in the 1-day-old fifth instar nymph it ranged from 10 to 17 μ in length (Fig. 9A) with a mean of 13.2 $\pm$ 0.9 μ. In the 6-day-old fifth instar nymph the range of the nuclear diameter was from 11 to 18 μ with a mean diameter 14.2 $\pm$ 1.1 μ (Fig. 9B). In the tepa-injected group it ranged from 14 to 25 μ (Fig. 9C) with a mean of 19.5 $\pm$ 1.4 μ. The difference in the means

between the 6-day-old control and the tepa-injected groups is significant ($P < 0.01$).

Autoradiography

The ratio of labelled to unlabelled nuclei was approximately 1:10 in the control and tepa-injected groups. However, the intensity of the labelling was different. There were more of heavily labelled nuclei and less of moderately labelled nuclei in the control than in the tepa-injected group (Table VIII).

Figure 7. Frequency distribution of fat body nuclei of fifth instar nymphs of S. gregaria according to their DNA contents in arbitrary units (AU). A, 1-day-old controls; B, 6-day-old controls and C, 6-day-old tepa-injected insects. Total number of nuclei measured per group was 100.

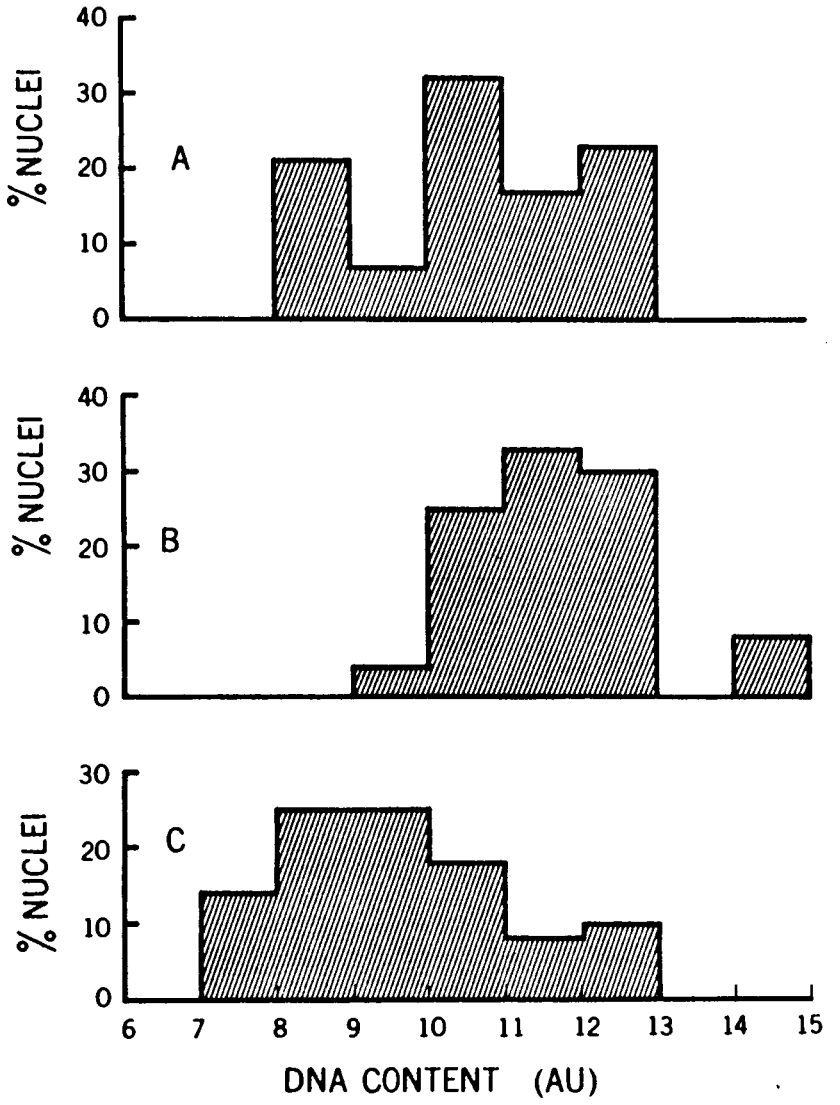

% NUCLEI
40
30
20
10
0
A
% NUCLEI
40
30
20
10
0
B
% NUCLEI
30
20
10
0
C
6
7
8
9
10
11
12
13
14
15
DNA CONTENT (AU)

Figure 8. Frequency distribution of fat body nuclei of fifth instar nymphs of S. gregaria according to classes of DNA-contents (degree of polyploidy). A, 1-day-old controls; B, 6-day-old controls and C, 6-day-old tepa-injected insects. Total number of nuclei measured per group was 100.

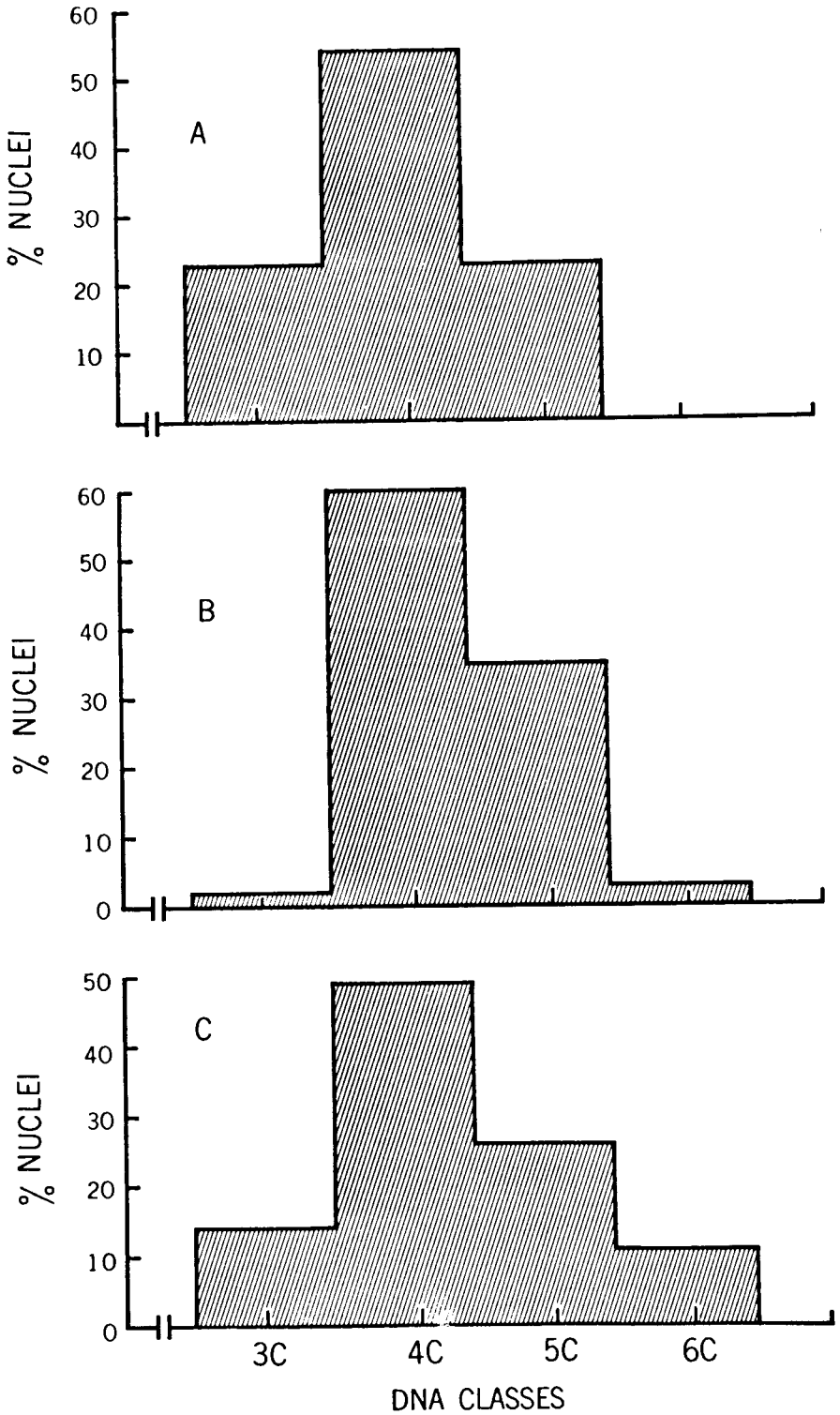

60
50
40
30
20
10
% NUCLEI
A
60
50
40
30
20
10
0
% NUCLEI
B
50
40
30
20
10
0
% NUCLEI
C
3C
4C
5C
6C
DNA CLASSES

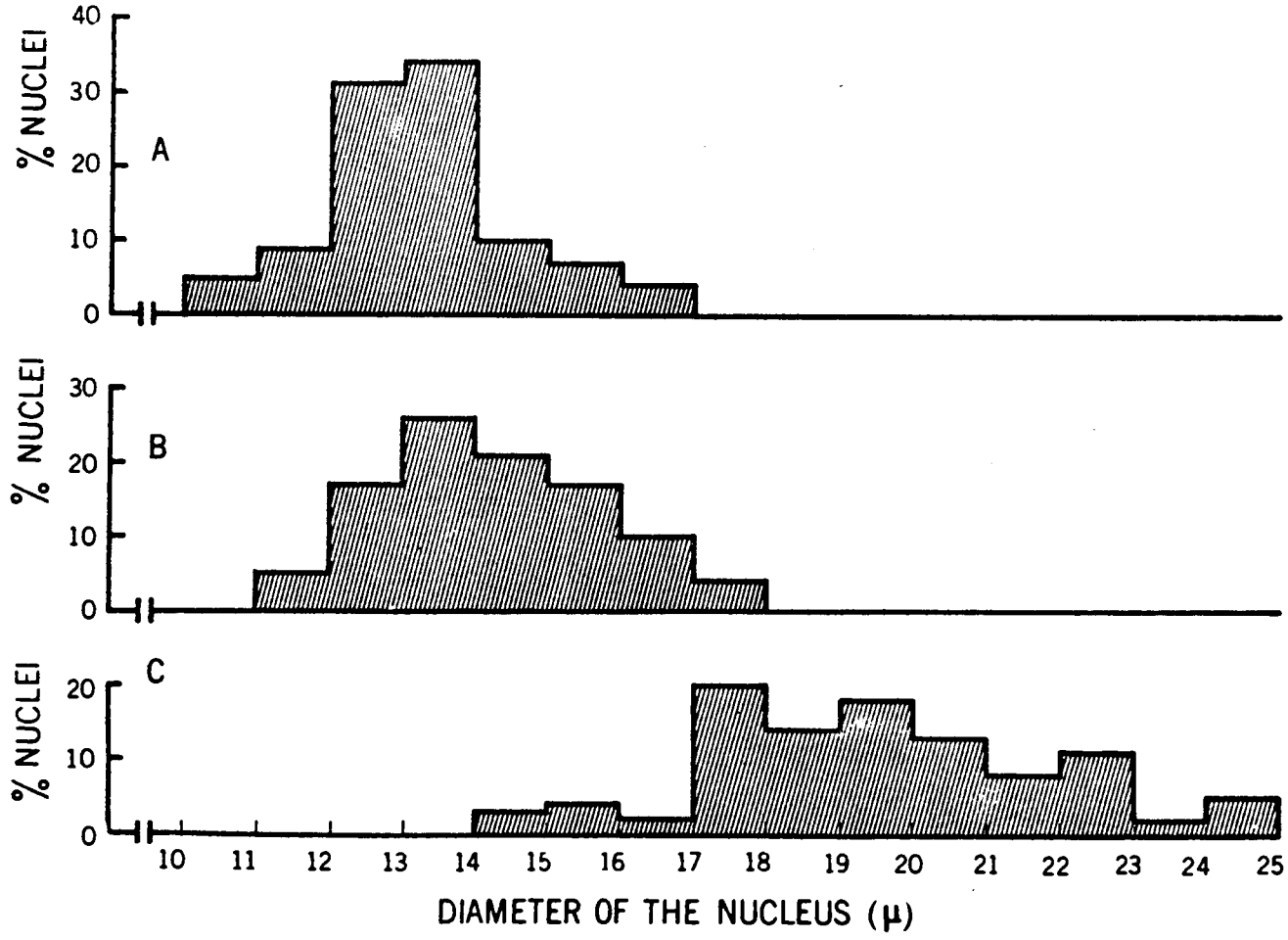
TABLE VII

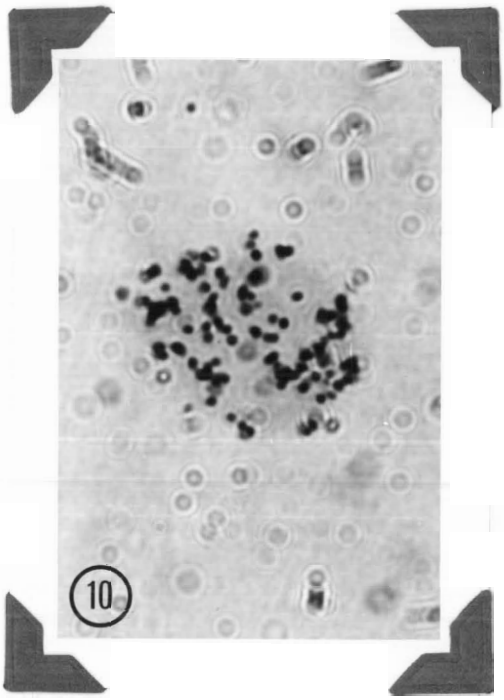
DNA classes (C) in fat body nuclei of 1-day-old controls, 6-day-old controls, and 6-day-tepa-injected fifth instar *S. gregaria*.

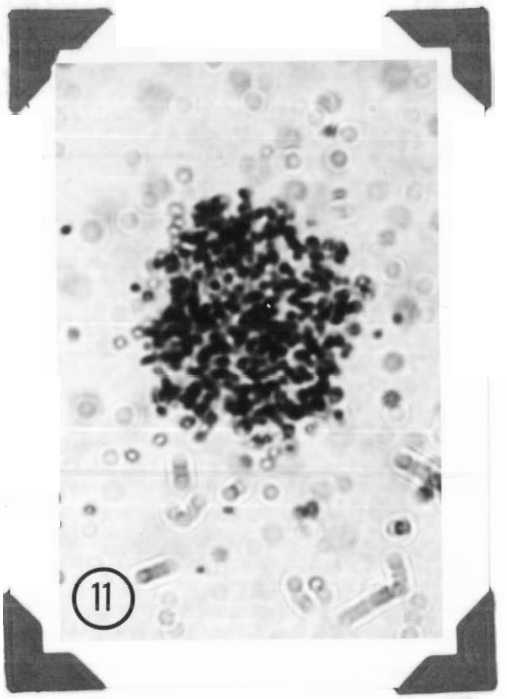
DNA Class	Percent of Nuclei		
	1 Day Control	6 Day Control	6 Day Treated
3C	23	2	14
4C	54	60	49
5C	23	35	26
6C	--	3	11

Each group consisted of 5 insects. Total number of nuclei measured per group was 100.

Figure 9. Frequency distribution of fat body nuclei in the fifth instar nymphs of S. gregaria according to their diameters in microns (μ). A, 1-day-old controls; B, 6-day-old controls and C, 6-day-old tepa-injected insects. Total number of nuclei measured in each group was 100.


% NUCLEI
40
30
20
10
0
A
% NUCLEI
30
20
10
0
B
% NUCLEI
20
10
0
C
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
DIAMETER OF THE NUCLEUS (μ)





Autoradiographs of fat body nuclei labelled with thymidine-H^3. Moderately labelled nucleus (Fig. 10). Heavily labelled nucleus (Fig. 11).

TABLE VIII

Effect of tepa on DNA synthesis in the fat body nuclei of female fifth instar nymphs of S. gregaria.

Group	Moderately Labelled Nuclei (%)	Heavily Labelled Nuclei (%)
Control	62	38
Tepa-injected	73	27

One hundred labelled nuclei from each group were examined to arrive at the percentages of moderately and heavily labelled nuclei. Slides were exposed in the dark for a period of 2 weeks prior to processing.

DISCUSSION

Polyploidy appears to be a normal occurrence in the somatic tissues of insects. It is well known that the dipterous salivary gland nuclei become polyploid by endomitosis. Geitler (1937) described 512-ploid nuclei in the salivary gland cells of the water spider Gerris lateralis. Berger (1938) observed that the nuclei of the epithelial cells of the ileum in Culex pipiens larvae underwent polyploidization by repeated internal divisions. From microspectrophotometric studies on Feulgen-stained nuclei Mittwoch et al. (1966) concluded that polyploidization occurs in the somatic cells of the honey bee Apis mellifera. Polyploid nuclei up to 32-ploid were found in the fat body nuclei of the fourth stage larvae of Rhodnius prolixus (Wigglesworth, 1967). This has been ascertained by counting the number of chromosomes. Wigglesworth attempted also to show the presence of intermediate nuclei of the triploid series by photometric measurements of Feulgen-stained nuclei, but the results were inconclusive since the measurements were variable and the number of nuclei measured was too small.

The results obtained in the present study indicate that the fat body cells are already polyploid in the fifth instar nymph of S. gregaria. Nuclei having from 3 to 5 times the DNA content of the germ cells are found in the 1-day-old fifth instar nymphs. Since there are nuclei in the 6C class, an increase in the

percentage of the nuclei in the classes 4C and 5C and a corresponding decrease in the class 3C in the 6-day-old fifth instar nymphs, it is evident that further polyploidization takes place in the fat body cells of the fifth instar nymphs during their growth.

Fox (1970) has recently described the DNA series in the somatic tissues of Locusta migratoria and S. gregaria adults. By microdensitometric methods Fox has shown that the DNA series do not correspond to a doubling series similar to that observed in the somatic tissue of seven Dermestes species (Fox, 1969). The lack of a doubling series in the somatic tissues of the locusts is probably due to under replication. The absence of doubling series and the presence of intermediate forms observed in the fat body cells of the fifth instar nymphs of S. gregaria could also be due to the fact that DNA synthesis might be occurring in some cells at the time of fixation.

Although there is a small increase in the percentage of 6 C nuclei in the tepa treated insects, it appears that tepa has a slight inhibitory effect on the process of polyploidization. The percentage of 3C nuclei is higher in the tepa-injected group than that of the 6-day controls, whereas the percentages of 4C and 5C nuclei are lower than those in the control group (Table VII). It is pertinent to mention here that tepa was injected when the nymphs were four days old.

If polyploidization takes place prior to this, then injection of tepa would not reveal its effect, if any, on DNA synthesis. It would have been more appropriate to inject tepa into 1-day-old fifth instar nymphs, but mortality was considerably high when tepa was injected at this phase of the fifth instar nymphs.

The results obtained in the autoradiographic study demonstrate beyond any doubt that DNA synthesis takes place during the later phase of the fifth instar nymph. Although the number of labelled nuclei in the controls and the tepa-injected insects is essentially similar, the intensity of labelling appears to be different in both the groups. In the controls 38% of the nuclei are heavily labelled whereas in the tepa-injected insects only 27% showed the same extent of labelling. Curiously enough the tepa-injected group showed an increase in the percentage of moderately labelled nuclei over that observed in the controls. Does this mean that repair mechanisms operate involving re-synthesis of the material removed by alkylation? The extent of inhibition of DNA synthesis is not considerable and this was not surprising since only 10% of the nuclei in the controls showed DNA synthesis.

The most significant effect of tepa is the increase in nuclear size. Harrington and Koza (1951) reported that irradiation of embryos of the grasshopper, Chorotophaga viridifaciata, with X-rays resulted in the enlargement of the

nuclear volume. These authors suggested that depolymerization of DNA could lead to an increase in the osmotic pressure and thus cause the nuclei to swell. Nair and Bhakthan (1969) observed swelling of the sarcoplasmic reticulum in the flight muscles of the housefly exposed to gamma-radiation. This has been attributed to changes in permeability induced by gamma-radiation. Whether the increase in the nuclear volume observed in the tepa-injected locusts is due to similar causes is not known.

Chapter 4

IV. EFFECT OF ALKYLATION OF DNA ON THE COLOR INTENSITY OF FEULGEN REACTION

INTRODUCTION

During the course of my investigations reported in the previous chapter it was observed that injection of tepa into fifth instar nymphs of S. gregaria resulted in a loss in the intensity of Feulgen staining in the fat body nuclei. A similar reduction in the intensity of Feulgen stain was also observed in the spermatogonial nuclei of tepa-injected fifth instar males. Hence it was assumed that the reduction in the intensity of Feulgen stain observed in the fat body nuclei of tepa-injected fifth instar female nymphs was not entirely due to reduction in the DNA content but due to interference in the Feulgen reaction. The following experiments were conducted to determine whether alkylation of DNA would prevent unmasking of the aldehyde groups necessary for Feulgen reaction.

MATERIALS AND METHODS

The following two experiments were carried out to investigate whether alkylation of DNA inhibits the intensity of Feulgen stain.

Experiment I

Squash preparations of the testes from the fifth instar nymph of S. gregaria were placed in 1% tepa solution in methanol for 15 or 60 minutes. The control preparations were placed in methanol for the same periods. The slides were then washed in distilled water and fixed in 10% neutral formalin. DNA content in AU of 20 Feulgen-stained nuclei from each of the control and tepa-treated preparations was determined as outlined in Chapter 3.

Experiment II

In this study the Feulgen reaction was carried out on a solution of DNA (Sigma). The method of staining DNA in solution was similar to that described by Hardonk and van Duijn (1964). To 0.2 ml of 0.5% DNA in distilled water was added 0.8 ml of a solution of 1% tepa in distilled water. The controls consisted of 0.2 ml of 0.5% DNA plus 0.8 ml of distilled water. Alkylation of DNA was carried out at 37° C for 1 hour. The control tubes were also incubated for the same period. After incubation 2 ml of 2N HCl (60° C) were added into each tube and the DNA hydrolyzed for 15 minutes at 60° C. Preliminary studies

showed that 15 minutes of hydrolysis was necessary for the development of maximum color after the addition of the Schiff's reagent. After hydrolysis the tubes were cooled rapidly and 0.3 ml of 35% NaOH and 0.1 ml of 15% solution of potassium metabisulphite were added into each tube. After thorough mixing in a vortex mixer, 1 ml of Schiff's reagent was added. After 3 hours the optical density of the color developed was determined in a Unicam SP 500 spectrophotometer at 560 nm. Five replicates each of the control and tepa-treated samples were used in this study.

TABLE IX

Effect of alkylation with tepa on Feulgen-revealed DNA content (AU) of spermatogonial nuclei of S. gregaria.

Nucleus No.	Control 15 minutes	Control 1 hour	Nucleus No.	Alkylated samples 15 minutes	Alkylated samples 1 hour
1	5.4	4.4	1	3.1	2.4
2	4.1	4.1	2	2.8	2.9
3	4.5	5.1	3	2.9	2.6
4	4.4	5.1	4	2.6	2.3
5	4.3	4.4	5	3.2	3.1
6	4.5	3.6	6	3.1	2.7
7	3.8	4.2	7	2.7	2.7
8	3.7	4.0	8	2.4	2.7
9	4.2	4.8	9	2.9	2.4
10	5.5	4.3	10	2.8	2.9
11	5.3	4.1	11	2.8	2.9
12	4.1	4.5	12	2.6	3.0
13	4.1	3.7	13	2.7	2.9
14	4.0	4.8	14	2.8	2.5
15	4.5	4.6	15	2.7	3.2
16	4.7	3.7	16	3.1	3.0
17	4.6	4.9	17	2.6	2.5
18	4.0	4.2	18	2.3	2.6
19	4.1	4.4	19	2.9	2.9
20	4.8	4.2	20	2.2	2.8
	$\bar{x}$S.D.± 4.43±0.48	4.35±0.42		2.76±0.24	2.75±0.22

Comparison of the means of the control and treated samples show that the differences are highly significant ($P < 0.01$). The t values for the 15 and 1 hour samples are 12.7 and 13.9 respectively.

TABLE X

Effect of alkylation of DNA on the color intensity of Feulgen reaction expressed as optical density at 560 nm.

Replicate	Control	Alkylated Sample
1	0.40	0.04
2	0.39	0.11
3	0.80	0.19
4	0.70	0.38
5	1.00	0.38
	$\bar{x}\pm$S.D. 0.658±0.235	$\bar{x}\pm$S.D. 0.220±.139

Comparison of the means of the control and alkylated samples showed that the differences are significant $P < 0.05$ ($t = 3.07$).

RESULTS AND DISCUSSION

The DNA value relative to the Feulgen stain was significantly lower in the tepa-treated spermatogonial nuclei than that of the controls (Table IX). Within 15 minutes of alkylation the DNA values were reduced to approximately 50%. No further reduction was observed after 1 hour of alkylation indicating the completion of alkylation within the first 15 minutes. The results of the experiments are in agreement. The optical density of the color developed in the alkylated samples was significantly lower than that of the control samples (Table X). These results demonstrate that alkylation of DNA inhibits recoloration of the Schiff's reagent.

It is established that aldehyde functions are responsible for the recoloration of the Schiff's reagent. Hence any reaction that involves blocking of the aldehyde groups or preventing the unmasking of aldehyde groups would lead to a reduction in the intensity of Feulgen stain. This may lead to erroneous conclusions in quantitative cytochemistry of DNA. The results of both experiments show that the reduction in the intensity of Feulgen stain in the alkylated samples is not solely due to lesser quantities of DNA but due to the fact that alkylation of DNA interferes with the Feulgen reaction.

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APPENDIX 1

Composition of medium A used for incubating fat body.

Constituent	mg/100 ml	Constituent	mg/100 ml
KCl	298	Glycine	65
$CaCl_2$	81	L-Histidine HCl	250
$MgCl_2$	304	L-isoleucine	10
$MgSO_4$	370	L-lysine HCl	125
NaH_2PO_4	110	L-methionine	10
Malic acid	67	L-phenylalanine	15
L-alanine	45	L-proline	35
L-arginine HCl	70	L-serine	110
L-asparagine	35	L-threonine	35
L-aspartic acid	35	L-tryptophan	10
L-cysteine HCl	8	L-tyrosine	5
L-cystine	2.5	L-valine	20
L-glutamic acid	60		
L-glutamine	60		

The solution also contained streptomycin sulfate (25 μg/ml) and penicillin G (250 units/ml) and the pH was adjusted to 6.5 with NaOH. To avoid precipitation, the $CaCl_2$ was added after mixing the amino acids with the other salts.

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