The Biosynthesis of Deoxyribonucleic Acid during the Life-Span of the Mosquito

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1. Previous results from this laboratory [Kao *et al.* (1976) *Biochem. J.* **154**, 471–480] demonstrated that a low-molecular-weight DNA species, 'sDNA', present in high concentrations during rapid larval growth of the yellow-fever mosquito, *Aedes aegypti*, may be an intermediate in DNA replication. We have now determined the life-span profiles of synthetic rates and the steady-state contents of sDNA and total DNA by radio-metric and biochemical procedures and by using mosquitoes grown and maintained under defined and controlled conditions, including axenic culture and synchronization of stages. 2. Our results showed exponential increases in DNA contents and synthetic rates which paralleled the changes in weight during larval growth. Of special importance was the finding that in rapidly-growing larvae and newly-emerged adult mosquitoes high contents and rates of synthesis of sDNA were correlated with increases in total DNA/organism, which suggests that sDNA content may be an index of DNA replication. In senescent mosquitoes a twofold increase in the synthetic rate of bulk DNA ('pDNA') without an increase in sDNA may reflect repair synthesis. 3. The significance of this investigation is that it may be the first quantitative and systematic description of the synthetic rates and contents of both DNA-replication intermediates and total DNA in an axenic, eukaryotic organism rather than a specific cell type. Equally important is that these findings describe DNA changes during growth and also during aging through senescence in an age-characterized organism.

The present investigation stemmed from the discovery that a low-molecular-weight DNA species found in the high-speed supernatant fractions of homogenates comprised at least one-third of the total DNA content during the period of most rapid growth of larvae of the yellow-fever mosquito (*Aedes aegypti*) (Lang *et al.*, 1965). Because of its occurrence in the supernatant fraction, it was termed soluble DNA, or 'sDNA', in contrast with the particulate (bulk) DNA, or 'pDNA'.

The isolation and characterization of sDNA and pDNA indicated that both have the same base composition, thermal-transition spectra, and buoyant density, but differ in size (Kao *et al.*, 1976). The results of various mixing experiments indicated that sDNA was not merely a nuclease degradation product of pDNA (Lang & Meins, 1966). In addition, pulse-chase experiments indicated that the labelled, newly-synthesized DNA was comprised of small-size fragments which later combined to form larger pieces in the size range of bulk DNA (pDNA) (Kao *et al.*, 1976). These results implicated sDNA as an intermediate in DNA replication.

Our efforts have been directed toward elucidation of the metabolic role of sDNA during the life-span of the mosquito. As an experimental model, the

mosquito is metabolically similar to, but has distinct advantages over, microbial and higher-animal systems, including other insects. It is a multicellular, differentiated organism with discrete synchronized developmental stages and can be grown and aged rapidly under controlled nutritional conditions. These advantages have enabled the correlation of a number of molecular phenomena with the developmental status of individual organisms.

There is a paucity of information on DNA metabolism during the life-span of an organism. Exceptions include measurements of DNA contents in *Drosophila melanogaster* (Church & Robertson, 1966), in *Tribolium confusum* (Devi *et al.*, 1963) and in *Aedes aegypti* by Lang *et al.* (1965) who also developed and validated their analytical methods for mosquito tissues. In none of these studies, however, were DNA synthetic rates also determined. The synthesis of DNA *in vivo* was measured in *Tribolium confusum* by Chaudhary & Lemonde (1966), but their data were derived from long-term incorporation rates in insects grown on a flour diet containing [¹⁴C]-thymidine rather than from short, initial rates obtained by using standard kinetic criteria.

In general, rates of DNA synthesis in insects have been measured by radioautographic methods which

cannot yield precise kinetic data. Moreover, most studies have been restricted to specific cells or organs, and in particular, the giant polytene chromosomes of Diptera. A comprehensive review of the extensive research on the synthetic activities of these giant chromosomes has been compiled by Berendes (1973). Although these investigations have been useful for the elucidation of molecular phenomena at the chromosome level, their relevance to DNA metabolism of the whole organism is limited.

The need for information concerning DNA metabolism of an age-characterized, eukaryotic organism during growth and also during aging prompted this investigation. Our objectives were to quantify systematically the changes in synthetic rates and steady-state contents of both sDNA and pDNA throughout the life-span of the mosquito.

Materials and Methods

Experimental organism and its culture

The experimental organism was the mosquito *Aedes aegypti* (Louisville), which has been colonized in this laboratory for over 15 years (approx. 200 generations). The larval period lasts approximately 7 days, and the pupal period lasts about 48 h under our standard conditions.

The biological ages of adult mosquitoes are as follows: 0–10 days, adult development; 10–25 days, maturity; 25–38 days; senescence; 38+ days, very old age. These periods were characterized from the survival curves for nine different populations grown and aged under our standardized conditions. The median survival time for these mosquitoes is 30 ± 2 days, which is also the time of highest mortality rate.

Standard culture. A standardized culture procedure using defined conditions was used to produce mosquitoes of all ages throughout the life cycle. Larval culture has been described by Kao *et al.* (1976).

Pupae were transferred to distilled water until they emerged as adults. Adult mosquitoes were fed from cotton pads moistened with 10% (w/v) sucrose in water. No blood meals were offered to the experimental colonies, and thus ovarian development was arrested. A temperature of 29°C was maintained throughout.

Axenic culture. Mass cultures of mosquito larvae were grown axenically on a defined medium as described by Lang *et al.* (1972). Axenic and standard (non-axenic) mosquitoes have the same rates of growth, pupation and adult emergence.

Materials

[2-¹⁴C]Thymidine (50.8–56 mCi/mmol) was obtained from Schwarz/Mann, Orangeburg, NY, U.S.A., and from Nuclear Dynamics, El Monte, CA, U.S.A. Crystalline thymidine (Sigma Grade) was

obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Protosol and Liquifluor (PPO*/POPOP/toluene concentrate) were from New England Nuclear Corp., Boston, MA, U.S.A. Other chemicals were of reagent grade, and double-distilled water was used. Whatman GF/A glass-fibre discs (2.4 cm) were used in the collection of samples.

Continuous labelling

Approximately 60 newly hatched mosquito larvae were added to 250 ml of either standard or axenic media containing 1 μ Ci of [¹⁴C]thymidine. They were grown in the radioactive medium until removal for sampling or until pupation, when the pupae were transferred to distilled water from which adult mosquitoes emerged. They were maintained throughout adult life as described above.

Pulse labelling

Rinsed larvae were pulsed for 1 h as a routine by placing them into 10 μ M-thymidine containing 1 μ Ci/ml of [¹⁴C]thymidine. Adult female mosquitoes of known age were cold-inactivated before injection. *Aedes* Ringer solution (0.13 M-NaCl/4.7 mM-KCl/1.9 mM-CaCl₂/7.0 mM-KH₂PO₄, adjusted to pH 6.8 with KOH) (1 μ l) containing 0.002 μ Ci of [¹⁴C]-thymidine was injected into each mosquito through the membranous region between the first and second thoracic spiracles. Injection needles were made from Pasteur pipettes (Medglass) pulled out to very fine points (approx. 0.1 mm diam.) over a microflame. Injected mosquitoes were kept at 29°C for 45 $\pm$ 15 min and were then quickly inactivated at 0°C, weighed and homogenized. The precision of the procedure is indicated by the fact that the mean and S.E.M. of the injected radioactivity calculated from 26 separate experiments was $58\,717 \pm 3614$ d.p.m./100 mg body weight.

Standard preparation of samples

Cold-inactivated larvae or adult mosquitoes were homogenized in 0.25 M-sucrose/0.02 M-EDTA, pH 8.0, by using an all-glass Ten Broeck homogenizer. A portion of the homogenate (5–10%, w/v) was centrifuged for 30 min at 100 000g at 4°C in a SW 50.1 swinging-bucket rotor in a Beckman model L ultracentrifuge. The supernatant was removed, and the pellet was resuspended in homogenizing medium and recentrifuged under the same conditions. The washing was combined with the first supernatant. The fractions were acid-precipitated by the addition of trichloroacetic acid to a final concentration of 5% (w/v) and centrifuged at 5500 rev./min

* Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazol-2-yl)benzene.

for 20 min to separate the acid-insoluble and acid-soluble materials. The pelleted fractions were solubilized in 1 ml of Protosol at 55°C, and 10 ml of scintillation fluid were then added. Protosol was also added to portions of the homogenate and to acid-soluble fractions and scintillation fluid added after incubation. All procedures before Protosol treatment of the samples were carried out at 0–4°C.

In some experiments the acid precipitates were filtered on to glass-fibre discs and rinsed with 12 ml of 5% (w/v) trichloroacetic acid, 16 ml of 95% ethanol, and 10 ml of ether, were dried, and counted for radioactivity in 5 ml of scintillation fluid. The recovery of radioactivity in the fractions relative to the radioactivity of the homogenate was determined in all cases.

On the basis of the results of Kao *et al.* (1976), the radioactive acid-precipitable material of the supernatant fraction was defined as 'sDNA' and that of the particulate fraction as 'pDNA'.

Liquid-scintillation counting

Single-phase system. Scintillation fluid (42 ml of Liquifluor/litre of toluene) was added to glass vials containing samples digested in Protosol. The vials were counted for radioactivity in either Nuclear-Chicago Unilux II or Mark II scintillation counters by using channels-ratio settings.

Disc counting. Proportionality of radioactivity and sample size, and optimal drying time were determined for the disc counting of homogenates and acid-soluble fractions. Non-specific adsorption of label was <1%. Discs were counted for radioactivity in 5 ml of scintillation fluid. The counting efficiencies of different fractions were determined relative to the single-phase system so that the radioactivity of disc preparations in c.p.m. could be converted to d.p.m. The counting efficiencies for the fractions from mosquitoes of different ages ranged from 23 to 67%.

Statistical analysis

The data have been computed so that results are expressed on the basis of 100 organisms or of 100 mg body weight to elucidate changes in individuals in which weight increases during growth may affect concentrations. All statistical procedures were carried out according to the standard methods given in Snedecor (1946) and facilitated by the use of a Hewlett-Packard mini-computer.

Results

Experimental procedures

Essential to this study was the development of a sensitive and precise method by which the steady-state DNA contents were quantitated throughout

the life-span of the mosquito. Continuously labelled larvae and pre-labelled adult mosquitoes were obtained by growing newly hatched larvae in a radioactive medium until pupation. Larval development, pupation, and adult-emergence rates were the same as for mosquitoes cultured in non-radioactive medium. Although survival curves were not established for the radioactive populations, mosquitoes 40 days of age or older were easily obtained. There were no apparent deleterious alterations observed in the continuously labelled larvae or adults.

It was of utmost importance in evaluating DNA metabolism over a time-period to ascertain that the observed changes were inherent in the experimental organism and did not reflect changes in the associated bacterial population. Larvae grown on a standard culture medium derive their nutrients from a bacterial infusion and consequently contain intestinal microflora. Bacteria did not present a problem in experiments involving colorimetric determinations as their contribution was negligible, but their presence affected radiometric evaluations. For this reason, comparisons were made between axenic- and standard-cultured (non-axenic) mosquito larvae under different experimental conditions. Distinct quantitative differences were found in uptake and incorporation of radioactive label by the differently cultured larvae. These differences were present in continuously labelled larvae from the same hatch as well as from several hatches (Fig. 1a). In addition, pulsed axenic larvae exhibited a rate of incorporation three times more rapid than the standard larvae and maintained linearity of both uptake and incorporation throughout a 4 h pulse (Fig. 1b). By contrast, standard larvae had a linear rate only up to 2 h. These findings formed the basis for using only axenically-cultured larvae in subsequent experiments. However, standard-cultured adult mosquitoes were used, since the precursors were administered by injection and thus circumvented the intestinal microflora. Moreover, the diet of the adult mosquito consisted of sucrose rather than a bacterial infusion.

DNA metabolism during larval development

(1) **Steady-state DNA contents.** Axenic larvae continuously labelled with [¹⁴C]thymidine from the time of hatching showed exponential increases in DNA content which paralleled the increase in weight for the first 4 days (Fig. 2). The doubling time for DNA during the first 4 days was 10.5 h according to a least-squares calculation. Although the pDNA component was approximately ten times that of sDNA, the two profiles were similar. There was also a close correlation of the sDNA and pDNA profile with that of DNA content determined previously by a colorimetric procedure (Lang *et al.*, 1965) (Fig. 3).

Fig. 1. Differences in uptake and incorporation of [¹⁴C]thymidine by continuously labelled or pulse-labelled axenic and standard (non-axenic) larvae

Uptake is defined as the radioactivity in the homogenate, and incorporation is defined as the radioactivity in the acid-insoluble fraction. Samples were taken at the times indicated and processed as described in the Materials and Methods section. (a) Larvae from different hatches were continuously labelled by culturing under axenic or standard (non-axenic) conditions in media supplemented with [¹⁴C]dT. Results are expressed as radioactivity (d.p.m.)/organism. (b) Larvae, 3 days of age, cultured under standard or axenic conditions were pulsed in 10 μ M solutions of dT containing 1 μ Ci/ml of [¹⁴C]dT. Results are expressed as radioactivity (d.p.m.)/100 mg larval weight. Each point with vertical bar represents the mean $\pm$ S.E.M. of five to nine samples for standard larvae, and of eight to eleven samples for axenic larvae. Bars are omitted if the S.E.M. is less than the size of the point. $\blacktriangle$, Standard uptake; $\triangle$, standard incorporation; $\bullet$, axenic uptake; $\circ$, axenic incorporation.

A marked lag in both profiles occurred between days 4 and 5.

(2) *Time-course of thymidine uptake and incorporation.* Larvae, 3 and 5 days old, were pulsed to determine their rates of uptake and incorporation of [¹⁴C]thymidine into the acid-insoluble fractions and thereby establish a pulse time for routine use. Linear rates were observed for 1 h without a lag phase (Fig. 4). The rate of uptake by 5-day larvae was 1.7 times greater than the 3-day larvae, but the rate of incorporation was 1.9 times more rapid in the 3-day group. In 3-day larvae the rate of incorporation into both sDNA and pDNA continued linearly for 4 h (Fig. 5).

(3) *Synthetic rates.* The capacity for DNA synthesis which was determined by the measurement of synthetic rates can be evaluated from two different aspects: (a) the synthetic capacity per organism, and (b) the synthetic capacity per unit of weight, adjusted for uptake. The profile of synthetic rates for both sDNA and pDNA per organism increased exponentially to maximal values by 4–5 days and resembled the profile of steady-state values (Fig. 6). After 5 days, however, when body weight reaches a plateau, the rates decreased. For pDNA, the synthetic rate per

unit of weight adjusted for uptake decreased linearly from day 1 of larval hatch to pupation (Fig. 7). By contrast, this rate for sDNA increased to a peak at day 5 and then decreased. By day 7 just before pupation, little synthesis of both sDNA and pDNA occurred.

DNA metabolism in the aging adult mosquito

(1) *Kinetics of thymidine incorporation in mature and old mosquitoes.* Linearity of thymidine incorporation occurred at the same rate for both age groups and continued for 90 min in mature, 10-day mosquitoes, and for 45 min in old, 40-day mosquitoes (Fig. 8). A 45 min pulse time was therefore used as a routine in subsequent experiments.

(2) *Synthetic rates.* The high rates of synthesis of sDNA and pDNA found in newly-emerged, 1-day adult mosquitoes decreased 77–91% by day 10 (Fig. 9a). Thereafter the rate of pDNA synthesis in old mosquitoes increased twofold, but sDNA synthesis was essentially constant. The changes in pDNA synthesis were statistically significant (Table 1).

(3) *Contents of DNA species in pre-labelled adult*

Fig. 2. DNA contents throughout the larval stage of the mosquito

Axenic larvae continuously-labelled with [^{14}C]dT were sampled at 24 h intervals. Pupation occurred between 6 and 8 days. Fresh weight and content of radioactive DNA species are plotted semi-logarithmically. Each point with vertical bar represents the mean $\pm$ S.E.M. of eight to eleven samples from three or four different hatches. Bars are omitted if the S.E.M. is less than the size of the point. From 68 samples there was an overall recovery of $99.3 \pm 0.87\%$ (radioactivity of all fractions/homogenate radioactivity $\times 100$). $\bullet$, sDNA+pDNA; Δ , pDNA; $\blacksquare$, sDNA; $\circ$, fresh weight.

mosquitoes. Mosquitoes, which were continuously pre-labelled with [^{14}C]thymidine as larvae, were sampled at different stages of the adult life-span, and differential changes were observed (Fig. 9b). The relatively high sDNA content decreased one-third by day 10, reached a plateau and then decreased again in the very old, 40-day mosquito (Table 2). By contrast, the pDNA content increased slightly after day 10 and was constant thereafter. The acid-soluble fraction decreased after day 10 with a further marked decrease in the very old, 40-day mosquito.

Discussion

The discovery of sDNA, a low-molecular-weight DNA species found in high concentrations in rapidly growing mosquito larvae, led to a series of investigations in our laboratory which implicate it as a replication intermediate in DNA synthesis

Fig. 3. Two different measurements of DNA contents throughout the larval stage of the mosquito

The steady-state DNA contents (sDNA+pDNA) determined radiometrically by continuous labelling of larvae with [^{14}C]dT are plotted with the DNA contents determined by a colorimetric method (Lang *et al.*, 1965). $\bullet$, Radiometric data; $\circ$, colorimetric data.

Fig. 4. Age comparison of [^{14}C]thymidine uptake and incorporation by larvae

Axenic larvae were pulsed in [^{14}C]dT for different times up to 90 min. Uptake and incorporation are defined in the legend to Fig. 1. (a) Uptake. For 3-day larvae the rate was 1.16×10^4 d.p.m./h per 100 mg and the correlation coefficient of determination (r^2) was 0.989. For 5-day larvae the rate was 2.00×10^4 d.p.m./h per 100 mg, and r^2 was 0.950. (b) Incorporation between 0–1 h (percentage of uptake). For 3-day larvae the rate was 33.4% /h, and r^2 was 1.00. For 5-day larvae the rate was 18.1% /h, and r^2 was 0.995. $\bullet$, 3-day larvae; $\circ$, 5-day larvae.

Fig. 5. Time-course of sDNA and pDNA synthesis by 3-day larvae

Axenic larvae, 3 days of age, were pulsed up to 4 h in [^{14}C]dT. Samples were taken at the indicated times. For pDNA the rate of incorporation was 4.75×10^3 d.p.m./h per 100mg, and r^2 was 0.990. For sDNA the rate of incorporation was 2.43×10^2 d.p.m./h per 100mg, and r^2 was 0.984. ●, sDNA+pDNA; △, pDNA; ■, sDNA.

Fig. 6. DNA synthetic rates/organism throughout the larval stage of the mosquito

Axenic larvae were pulse-labelled 1 h in [^{14}C]dT. The results, plotted semi-logarithmically, are expressed as radioactivity incorporated/organism. Each point with vertical bar represents the mean $\pm$ S.E.M. of eight to eleven samples from three or four different hatches. From 64 samples there was an overall recovery of radioactivity of $94.5 \pm 0.96\%$. ●, sDNA+pDNA; △, pDNA; ■, sDNA.

Fig. 7. DNA synthetic rates/100mg larval weight throughout the larval stage of the mosquito

Values represent incorporated radioactivity as a percentage of homogenate radioactivity obtained from the same experiments described under Fig. 6. △, pDNA; ■, sDNA.

Fig. 8. Kinetics of [^{14}C]thymidine incorporation in mature and old adult mosquitoes

Adult mosquitoes, 10 and 40 days of age, were pulse-labelled with [^{14}C]dT. Values are the percentages of homogenate radioactivity incorporated into acid-insoluble material. The incorporation rate for 45 min was 0.0356%/min for 10-day mosquitoes, and 0.0369%/min for 40-day mosquitoes. ●, 10-day mosquitoes; ○, 40-day mosquitoes.

(Kao *et al.*, 1976). Such intermediates have been found in simpler systems using prokaryotes and cell cultures, but the finding of a possible replication intermediate *in vivo* in this multicellular eukaryote had special significance in that it provided an opportunity to quantify and describe systematically the synthetic rates and contents of both DNA-replication intermediates and total DNA during the entire life-span.

An important aspect of the present research involved the development of a new procedure for the continuous radioactive labelling of DNA *in vivo*. The importance of such a procedure to determine the steady-state DNA content was pointed out by Selman & Kafatos (1974) who found that thymidine administered to silkworm pupae by pulse-labelling disappeared from the nucleotide pool in 2 to 4 h, and thus this method is applicable only for determining synthetic rates. Continuous administration is needed when DNA contents are monitored over a longer period. Our continuous-labelling procedure enabled the quantitative and precise determination of the changes *in vivo* in concentrations of sDNA and pDNA throughout the life-span.

Since mosquito larvae can only be fed and not injected with the radioactive precursor, the determination of rates of both uptake and incorporation was essential. No lag times were observed in either parameter, which indicated the rapid transport of the precursor.

Our findings are consistent with the concept that sDNA is an intermediate in replicative DNA synthesis. Evidence to support this view is that during periods of active growth such as early larval and adult development, a high content and high rates of synthesis of sDNA accompanied increases in total DNA and cell mass. For these reasons we believe that the sDNA content may be an index of DNA replication. It should be pointed out, however, that sDNA content cannot be considered a specific measure of cell proliferation in insect larvae in which DNA synthesis also results in polyploidy or polyteny.

The patterns of DNA synthetic rates and steady-

Fig. 9. Changes in synthetic rates and contents of DNA throughout the adult stage of the mosquito

Synthetic rates were determined by pulse-labelling adult mosquitoes with [^{14}C]dT. Steady-state contents were determined from the incorporated radioactivity of mosquitoes which were continuously pre-labelled with [^{14}C]dT during larval development and allowed to develop into adults. The basis of expression was the percentage of homogenate radioactivity incorporated into acid-insoluble material. Relative rates and contents were based on the 10-day values of 100%. Each point with vertical bar represents the mean $\pm$ s.e.m. of three to nine samples. (a) Synthetic rates. The actual 10-day values/45 min were: pDNA, 1.47%; sDNA, 0.384%. Recovery of radioactivity was $99.3 \pm 1.72\%$ from 26 different samples. (b) Steady-state contents. The actual 10-day values were: pDNA, 67.7%; sDNA, 6.31%; acid-soluble fraction, 16.9%. Recovery of radioactivity was $92.1 \pm 1.79\%$ from 30 different samples. Δ , pDNA; $\blacksquare$, sDNA; $\circ$, acid-soluble fraction.

Table 1. Differential synthetic rates of DNA species in the aging adult mosquito

Mosquitoes of 10 (mature) and 40 (old) days of age were compared. Values are the means $\pm$ s.e.m. of five samples from each age group. '% of homogenate' means the recovery of radioactivity in the fraction relative to that of the homogenate. This and the other expressions of synthetic rate refer to radioactivity incorporated into acid-insoluble material in 45 min.

Fraction	Basis of expression	Synthetic rates		Relative change (old/mature)	Significance level, $P <$
		Mature adult	Old adult		
pDNA	(% of homogenate)	1.47 ± 0.110	2.82 ± 0.408	1.92	0.010
	(d.p.m./weight)	917 ± 19	1946 ± 281	2.12	0.005
	(d.p.m./organism)	2182 ± 45	4054 ± 585	1.86	0.010
sDNA	(% of homogenate)	0.384 ± 0.053	0.300 ± 0.043	0.781	0.150
	(d.p.m./weight)	239 ± 29	211 ± 34	0.883	0.300
	(d.p.m./organism)	569 ± 69	440 ± 72	0.773	0.150

Table 2. *Differential concentrations of DNA species in the aging adult mosquito*

Values are the means $\pm$ S.E.M. from three samples for 10-day, nine for 30-day, and six for 40-day adult mosquitoes. '% of homogenate' means the recovery of radioactivity in the fraction relative to that of the homogenate.

Age comparison	Fraction	Basis of expression	Concentration of DNA species in:		Relative change (very old/mature)	Significance level, $P <$
			Mature adult (10 days)	Very old adult (40 days)		
10 and 40 days	pDNA	(% of homogenate)	67.7 $\pm$ 2.38	87.8 $\pm$ 2.57	1.30	0.0005
		(d.p.m./weight)	4308 $\pm$ 415	5506 $\pm$ 872	1.28	0.200
		(d.p.m./organism)	6627 $\pm$ 638	6798 $\pm$ 1077	1.03	0.475
	sDNA	(% of homogenate)	6.31 $\pm$ 0.702	3.87 $\pm$ 0.100	0.613	0.0005
		(d.p.m./weight)	398 $\pm$ 39	240 $\pm$ 34	0.603	0.0125
		(d.p.m./organism)	612 $\pm$ 59	296 $\pm$ 42	0.484	0.0025
30 and 40 days	sDNA		Old adult (30 days)	Very old adult (40 days)	Very old/old	
		(% of homogenate)	6.79 $\pm$ 0.247	3.87 $\pm$ 0.100	0.570	0.0005
		(d.p.m./weight)	356 $\pm$ 39	240 $\pm$ 34	0.674	0.050
		(d.p.m./organism)	463 $\pm$ 51	296 $\pm$ 42	0.640	0.025

state contents observed in our experiments reflect a developmental sequence characteristic of holometabolous insect larvae in which the early larval period is dominated by growth of larval cells and the later phase by proliferation of imaginal disc cells (Anderson, 1972; Gehrig & Nöthiger, 1973). The increasing DNA content found during early larval life may be correlated with the development of polyploidy and polyteny in differentiated and functional larval cells which are generally histolysed during metamorphosis. The data demonstrate that the exponential increase in DNA content was interrupted at 4–5 days when the capacity for DNA synthesis was maximal. This lag may represent a change in the hormonal environment, as demonstrated in other insect species (Doane, 1973), but information on hormone titres in the mosquito is not available.

The increase in DNA content between day 5 and 6 may reflect both accelerated mitotic activity of imaginal discs and the growth of specific larval tissues such as heart, brain and fat body that are retained in the adult. By day 6, when the DNA content reached a maximum, low synthetic rates were found, and may indicate the beginning of metamorphosis. Further, the decrease in DNA content in 7-day-old, pre-pupal larvae correlates with the loss of polyploid cells of the mid-gut epithelium and replacement by diploid cells that occurs at this time (Trager, 1937).

The adult life-span of the mosquito is divided into several developmental stages based primarily on survival data. During the first 10 days that involve post-emergence maturation or completion of metamorphosis, changes in rates of protein synthesis and of several enzyme activities reflect a continuation of adult development (Lang & Stephan, 1967; Smith,

1971; Mills & Lang, 1972). By day 10 the organism is considered a mature adult and for that reason 10 days was chosen as the reference age to evaluate the relative changes in DNA metabolism. The time of most rapid mortality, 25–38 days, involves senescent changes, and mosquitoes older than that are very old survivors.

Our findings underscore the fact that the newly-emerged adult mosquito is not a mature organism since high rates of DNA synthesis were found in 1-day mosquitoes but dropped to low values by day 10 with a similar decrease in sDNA synthesis. Relatively high-steady-state sDNA contents were also found in the young adults.

Evidence of active DNA synthesis in newly-emerged adult mosquitoes has been noted by other investigators. Active mitoses and an increase in cell number were found in the follicular epithelium of the ovary of *A. aegypti* immediately after emergence from the pupa (Laurence & Simpson, 1974), but the rate of division was decreased after 24–48 h unless ovarian development was stimulated by a blood meal. The mosquitoes used in our experiments were fed entirely on sucrose, and ovarian development was arrested throughout adult life (Christophers, 1960); therefore DNA synthesis after maturation could not be related to mitotic activity of the follicular epithelium. On the other hand, our results may be related to radioautographic evidence of continued DNA synthesis in 13- and 23-day-old mature mosquitoes by Sharma *et al.* (1970), who found labelled nuclei in cells of gut epithelium, fat body, oenocytes and Malpighian tubules.

The total radioactivity incorporated into sDNA-plus-pDNA was constant after 5 days of adult life

in continuously pre-labelled mosquitoes, which is in agreement with the previous results determined by the diphenylamine reaction (Lang *et al.*, 1965). However, our present method revealed differential changes in sDNA and pDNA contents, for there was a decrease in sDNA concomitant with an increase in pDNA. Also, in old mosquitoes there was little residual radioactivity in the acid-soluble fraction.

Of particular interest was the increased rate of synthesis of pDNA, but not sDNA, in old mosquitoes relative to that of 10-day-old, mature mosquitoes. The age profile for pDNA synthesis in adults is correlated with that for protein synthesis, which increased in very old (35–40-day) mosquitoes (Smith, 1971). An additional correlation is the increased synthetic rates of RNA found in aging adult *Oncopeltus fasciatus* (McDowell *et al.*, 1974).

Three possibilities could explain the increased rates of DNA synthesis in senescence: (1) cell division and turnover; (2) the development of polyploidy or polyteny in old tissues; and (3) acceleration of repair synthesis. Although the mature mosquito has been considered a post-mitotic organism, the possibility that some cells are capable of replication cannot be ignored. The evidence of increased DNA content in nuclei of cells of aging *Drosophila* (Samis *et al.*, 1971; Bozcuk, 1972) in the absence of somatic mitosis was interpreted by Bozcuk (1972) to be the result of the formation of polytene nuclei in mid-gut cells, but there is no information concerning this possibility in mosquitoes.

Our belief is that in the old mosquito the increased pDNA synthesis concurrent with a low sDNA content represents repair or turnover, rather than replicative synthesis. Further investigation is needed to distinguish these possibilities.

It is increasingly obvious that renewal mechanisms are operative at organelle and molecular levels (Goss, 1970), and experimentation substantiates the belief that repair replication of DNA may be a normal occurrence in the life history of cells in response to sub-lethal exposure to a variety of environmental stimuli. The formation of thymine dimers in DNA by ionizing radiation and the attendant repair processes have been discussed in great detail by a number of investigators (Painter, 1972; Lett *et al.*, 1972; Shaeffer & Merz, 1972; Cleaver, 1972). The concept that a DNA-repair mechanism is a normal event in non-irradiated cells and may be accelerated in old age due to an accumulation of damage is gaining experimental support. It can be hypothesized that the increased rates of synthesis of several macromolecules that have been observed in old organisms are an adaptive response to the stress of old age and that the senescent mosquitoes may represent a highly-selected population of those organisms capable of such response and which are thus able to survive to a very old age.

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