A Deoxyribonucleic Acid-Replication Intermediate in the Growing Mosquito

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In previous experiments on growth and aging in the yellow-fever mosquito, *Aedes aegypti*, a low mol.wt. (500000) DNA species was found in the supernatant fraction after ultracentrifugation of homogenates of rapidly-growing larvae. This DNA species, 'sDNA', constituted 30-40% of total DNA in 2-4-day-old larvae, but was less than 5% in older larvae, pupae and adults. We have now isolated and characterized sDNA and initiated experiments to determine its metabolic role. Isolated sDNA has the same physical and chemical characteristics as bulk DNA, 'pDNA', and differs only in size. In CsCl isopycnic centrifugation the buoyant densities of sDNA and pDNA were 1.700 and 1.697 g/cm³ respectively. The 'melting' temperature of both DNA species was 84°C. Base compositions calculated from these data and other methods were 38.9 mol% of guanine-plus-cytosine for sDNA, and 38.5 for pDNA. Also, the size of newly-synthesized DNA was investigated by pulse-labelling and pulse-chase experiments. In neutral sucrose gradients the labelled DNA component after a 2 h pulse had a sedimentation coefficient of about 8S, but after a 4 h pulse sedimented in a broad band from 10-19S. In alkaline sucrose gradients a single peak around 7S was observed for pulse times up to 4h. After a 6h pulse and a 1 day 'chase', labelled DNA species had sedimentation coefficients ranging from 10-15S in alkaline sucrose, and after a 2-day 'chase' the values were 17-31S, similar to those of pDNA under alkaline conditions. These results suggest that sDNA represents an intermediate form in the replication of DNA in mosquito larvae.

There is evidence that DNA replication in eukaryotic cells is a discontinuous process which involves the synthesis of small pieces of DNA and their subsequent linkage to form bulk DNA (Burgoyne, 1972; Hayton *et al.*, 1973; Taylor *et al.*, 1973). In this respect, DNA replication in eukaryotes is similar to that in bacteria (Geider & Hoffmann-Berling, 1971; Okazaki *et al.*, 1968). However, details of the process are still obscure, and there are differing reports on the nature of the intermediate fragments (Brewer *et al.*, 1974; Louarn & Bird, 1974; Taylor, 1974; Gautschi & Clarkson, 1975).

A DNA species of low mol.wt. (500000) was found in high concentration in rapidly growing larvae of the yellow-fever mosquito (*Aedes aegypti*) (Lang *et al.*, 1965). This DNA, which separated into the supernatant fraction after ultracentrifugation of larval homogenates, was called 'sDNA' in contrast with the pelleted, or bulk DNA, which was called 'pDNA'.

Subsequent experiments demonstrated that sDNA was double-stranded and apparently not a product of the enzymatic degradation of bulk DNA (Lang & Meins, 1966). The most significant finding was that sDNA represented 30-40% of the total DNA in

2-4-day-old rapidly-growing larvae, but less than 5% in older larvae, pupae and adults in which net growth has ceased.

In the present paper we report the isolation and further characterization of sDNA and pDNA and also the results of pulse-labelling and pulse-chase experiments to elucidate the mode of DNA replication. Our results indicate that sDNA may be an intermediate in the replication of DNA in mosquito larvae. A preliminary account of this work has been presented (Kao & Lang, 1971).

Experimental

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).

Standard culture. A standardized culture procedure using defined conditions was used to produce mosquito larvae at all stages. The procedure is outlined below.

Eggs stored at 29°C less than 1 month were synchronously hatched by agitating them in distilled water on a vortex mixer for 4min. Larvae which

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emerged within 15 min were transferred to culture pans containing distilled water with a mineral supplement corresponding to that used in axenic media. An aq. 10% (w/v) suspension of desiccated pig liver (Wilson and Co., Chicago, IL, U.S.A.) was added daily to maintain a bacterial infusion which was the principal source of nutrients for the growing larvae. A temperature of 29°C was maintained throughout.

Axenic culture. Mass cultures of mosquito larvae were also grown axenically on a defined medium as described by Lang *et al.* (1972). Axenic and standard mosquitoes have the same rates of growth and gross chemical composition.

Materials

[2-¹⁴C]Thymidine (45.5–53.6 mCi/mmol) and CsCl (optical grade) were obtained from Schwarz/Mann, Orangeburg, NY, U.S.A. Calf thymus DNA, salmon sperm DNA, and bovine pancreatic RNAase* and DNAase were from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and Pronase (grade B) was from Calbiochem, La Jolla, CA, U.S.A. ϕ X174 DNA (22.5S) was obtained from Miles Laboratories Inc., Kankakee, IL, U.S.A., and the *Micrococcus lysodeikticus* DNA was a gift from Dr. F. J. Bollum, Department of Biochemistry, University of Kentucky School of Medicine. The disodium salts of dAMP, dGMP and dCMP were from Sigma Chemical Co., St. Louis, MO, U.S.A. The following materials were also used: Sepharose 6B and Sephadex G-50 (Pharmacia, Uppsala, Sweden); Whatman GF/A, 2.4 cm glass-fibre discs; t.l.c. plates, Cellulose F (E. Merck, Darmstadt, West Germany); sodium dodecyl sulphate (95%) (Matheson, Coleman and Bell, Norwood, OH, U.S.A.). Other chemicals were of reagent grade, and glass-distilled water was used throughout.

Methods

Separation of sDNA and pDNA fractions. Cold-inactivated larvae were homogenized either in 0.25M-sucrose or in 0.15M-NaCl/0.10M-EDTA, pH 8.0, by using an all-glass Ten Broeck homogenizer. Homogenates (10–30%, w/v) were centrifuged for 30 min at 124000g at 4°C by using a SW39L swinging-bucket rotor in a Beckman model L ultracentrifuge. The supernatant was removed, and the pellet was resuspended in homogenizing medium and centrifuged again under the same conditions. The washing was combined with the first supernatant. All procedures were carried out at 0–4°C.

* Abbreviations: RNAase, ribonuclease; DNAase, deoxyribonuclease. SSC medium, 0.15M-NaCl/15 mM-trisodium citrate/0.1 mM-EDTA, pH 7.0; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazol-2-yl)-benzene.

Isolation of sDNA and pDNA. (1) sDNA. Sodium dodecyl sulphate was added to the supernatant fraction to make a 2% (w/v) solution, was then incubated 10 min at 60°C and then shaken with an equal volume of chloroform/butanol (24:1, v/v) for 25 min. After centrifugation at 10000g for 20 min the emulsion separated into an upper aqueous phase, a middle layer of denatured protein and a lower chloroform phase. The upper phase was removed and shaken with chloroform/butanol at least six times until there was no visible middle layer of denatured protein. It was then incubated at 37°C for 1 h with 125 µg of heat-treated RNAase/ml, shaken four more times with chloroform/butanol and extracted three times with an equal volume of ether. The solution was concentrated in dialysis tubing by pervaporation, applied to a column of Sepharose 6B and eluted with SSC medium. The first u.v.-absorbing peak, which contained the sDNA, was concentrated, and the amount of sDNA was measured spectrophotometrically at 260 nm assuming an absorbance of 20 (in a 1 cm light-path) for a 1 mg/ml solution.

(2) pDNA. The particulate fraction from the homogenate was resuspended in homogenizing medium, and sodium dodecyl sulphate was added to a concentration of 2% (w/v). The suspension was incubated at 60°C for 10 min and then at 37°C for 2 h with 2 mg of preincubated Pronase/ml. It was then shaken at least seven times with chloroform/butanol, and the pDNA was then further purified as described for sDNA.

Determination of base composition. (1) T.l.c. The perchloric acid-hydrolysis method of Wyatt (1951) which was adapted for t.l.c. by Holdgate & Goodwin (1964) was used. A 5 µl portion of hydrolysate (approx. 0.5 mg of mosquito sDNA or pDNA) was chromatographed. U.v.-absorbing zones were scraped from the plate and extracted with 0.1M-HCl for 90 min at 95°C. The molar concentration of each base was calculated by the differential-extinction technique (Bendich, 1957). An area on the layer adjacent to each zone was processed similarly and served as a blank absorbance correction. To validate our procedure, the base compositions of calf thymus DNA and salmon sperm DNA were also determined by this method and were within 3% of reported values (Jordan, 1960).

(2) Isopycnic centrifugation. Buoyant densities of DNA samples were determined by centrifugation to equilibrium in CsCl gradients. Starting solutions contained 1–2 µg of mosquito DNA/ml, 0.5 µg of *Micrococcus lysodeikticus* DNA/ml as a density marker, and sufficient CsCl to give a density of 1.710 g/cm³ as measured by refractive index. The solutions were centrifuged in a Beckman model E analytical ultracentrifuge at 44770 rev./min for 24 h at 20°C. U.v.-absorbance photographs were taken, and tracings were made with a Joyce/Loebl double-beam

automatic-recording microdensitometer. The buoyant density of DNA was calculated from equations given by Sueoka (1961), by using a value of 1.727 g/cm^3 for the density of the marker (Skinner, 1967). The equation given by Schildkraut *et al.* (1962) was used to calculate the mole fraction of guanine-plus-cytosine (G + C) from the buoyant density of each DNA sample.

(3) Thermal denaturation of DNA. Samples of sDNA and pDNA in SSC medium were diluted with SSC medium until their E_{260} was about 0.3. Cuvettes were tightly sealed to avoid evaporation of solvent. Temperature was regulated with a programmed water bath (Neslab Instruments) connected to a special, jacketed cuvette holder for the Zeiss PMQ II spectrophotometer. Temperature was increased at a rate of $1^\circ\text{C}/15\text{--}20\text{ min}$. E_{260} was recorded for each temperature and corrected for the thermal expansion of water. The temperature corresponding to the midpoint of the absorbance rise (T_m) was determined, and from it the mol fraction of guanine-plus-cytosine was calculated by the equation of Marmur & Doty (1962).

(4) Spectral analysis. The base composition of DNA was estimated by the u.v.-absorbance method of Hirschman & Felsenfeld (1966). Spectra of native DNA were determined at room temperature (22°C), and spectra of fully denatured DNA were obtained at 95°C with the same equipment used for thermal-denaturation studies. The adenine-plus-thymine content (mol %) was calculated by the 'hyperchromicity method' given by Hirschman & Felsenfeld (1966).

Newly-synthesized DNA. (1) Pulse-labelling and pulse-chase. Axenic larvae were pulse-labelled with $[2\text{-}^{14}\text{C}]\text{thymidine}$ as described in the Figure legends. The labelling medium was supplemented with the disodium salts of dAMP, dGMP and dCMP, each at a final concentration of $0.1\text{--}0.5 \text{ mM}$. The $[^{14}\text{C}]\text{thymidine}$ pulse was 'chased' by adding at least a 1000-fold excess of thymidine or by transferring washed larvae into fresh medium containing thymidine. Samples of larvae were washed, weighed and killed by freezing at -15°C .

(2) Determination of radioactivity. sDNA and pDNA fractions from ^{14}C -labelled larvae were separated as described above. The fractions were acid-precipitated by the addition of trichloroacetic acid to a final concentration of 5% (v/v), and the precipitates were filtered on glass-fibre discs. The discs were rinsed successively with 12 ml of 5% (w/v) trichloroacetic acid, 16 ml of 95% (v/v) ethanol and 10 ml of ether and then dried. Portions of the acid-soluble fractions were also spotted on to discs and dried.

The discs of all fractions were placed in 5 ml of either dioxane scintillation fluid (10 g of naphthalene, 1 g of PPO, 0.025 g of POPOP and dioxan to 100 ml), or toluene scintillation fluid (0.5 g of PPO, 0.03 g of

POPOP and toluene to 100 ml) and were counted in a Nuclear-Chicago Unilux II liquid-scintillation counter.

(3) Incorporation of radioactive thymidine into DNA. Larvae grown for 3 days in axenic culture medium containing $10 \mu\text{Ci}$ of $[^{14}\text{C}]\text{thymidine}/150 \text{ ml}$ were homogenized in $0.13 \text{ M-NaCl}/0.01 \text{ M-Tris}$, pH 7.0. The supernatant fraction from the centrifugation of a 20% (w/v) homogenate was combined with an equal volume of redistilled 90% (v/v) phenol. The aqueous phase was extracted three times with equal volumes of ether and then divided into three portions of 0.6 ml each. The first portion was untreated and served as a control. The second portion was incubated at 25°C for 90 min with 1 mg of DNAase and 0.3 ml of $0.3 \text{ M-sodium acetate}$ containing 0.015 M-MgSO_4 , pH 5.0, and then at 37°C for 15 h after the addition of 1 mg of preincubated Pronase. The third portion was incubated at 37°C for 90 min with 0.2 ml of RNAase solution ($200 \mu\text{g}$ of RNAase in 0.15 M-NaCl , pH 5.0) which had been pretreated at 80°C for 10 min. The mixture was further incubated at 37°C for 15 h after the addition of 1 mg of Pronase. Each portion was applied to a column ($1 \text{ cm} \times 30 \text{ cm}$) of Sephadex G-50 and eluted with $0.13 \text{ M-NaCl}/0.01 \text{ M-Tris}$, pH 7.0. Fractions (0.5 ml) were collected and the E_{260} was measured. Portions (0.2 ml) of the fractions were analysed for radioactivity in 5 ml of dioxane scintillation fluid.

(4) Localization of radioactivity in thymine. Purified sDNA obtained from $[^{14}\text{C}]\text{thymidine}$ -labelled larvae was combined with salmon sperm DNA as a carrier and then precipitated with an equal volume of cold 10% (w/v) trichloroacetic acid. The precipitate was dehydrated with 95% (v/v) ethanol, hydrolysed with 72% (w/v) HClO_4 and processed for t.l.c. as described above. Each u.v.-absorbing spot was collected and analysed for radioactivity in toluene scintillation fluid.

(5) Supernatant radioactivity in sDNA. The supernatant fraction and $150\text{--}200 \mu\text{g}$ of isolated sDNA/ml from ^{14}C -labelled larvae were centrifuged in CsCl solutions in separate tubes at 48000 rev./min for 25 h at 20°C in the SW50.1 rotor of a Beckman model L centrifuge. Fractions (15 drops) were collected from the bottom of the tube, and their E_{260} and radioactivity were determined. Fractions from the supernatant gradient were acid-precipitated before counting for radioactivity.

(6) Size of DNA species *de novo*. Total DNA was extracted from larvae pulse-labelled with $[^{14}\text{C}]\text{thymidine}$ for different periods of time. Special conditions of processing are detailed in legends to appropriate Figures and include the homogenization of live larvae in phenol or alkali to inhibit endogenous nuclease activity. Enzyme-grade sucrose was dissolved in SSC medium for neutral gradients or in $0.1 \text{ M-NaOH}/0.9 \text{ M-NaCl}/0.001 \text{ M-EDTA}$ for alkaline

gradients. Portions (0.2ml) of the DNA solutions were layered on to 5.0ml of linear 5–20% (w/v) preformed sucrose gradients in nitrocellulose tubes. Human haemoglobin, 4.6S (Jovin *et al.*, 1969) and ϕ X174 DNA, 22.5S, were used as markers in the neutral and alkaline gradients respectively. The samples were centrifuged at 22 500 rev./min for 15 h at 5°C in the SW39L rotor of the Beckman model L ultracentrifuge. Fractions (5 drops) were collected from the top by displacing the gradient with a 50% (w/v) sucrose solution. For the neutral gradients in which isolated DNA was used, 0.4ml of water and 5.0ml of dioxane were added to each fraction before counting for radioactivity. Fractions from the alkaline gradients, however, were precipitated with 1ml of cold 10% (w/v) trichloroacetic acid, and 0.9ml of a 0.2% egg albumin solution was added as carrier. The precipitates were collected on glass-fibre discs and their radioactivities determined. Sedimentation coefficients were calculated by using the equation of Martin & Ames (1961).

Results

Isolation of mosquito DNA species

A method for the isolation of sDNA and pDNA from mosquito larvae was developed which yielded 0.17mg of sDNA and 0.46mg of pDNA/g of larvae. The concentration of total isolated DNA was therefore 0.63mg/g of larvae, which was 63% of the value for total DNA in homogenates that was determined in earlier experiments by Lang *et al.* (1965). These isolated preparations, which were free from protein and RNA, were used in the characterization studies.

To obtain maximal yield, different conditions were developed for the DNA preparations analysed on sucrose gradients. On the other hand, the procedures used to isolate DNA for physicochemical studies were designed to obtain maximal purity.

Table 1. Guanine-plus-cytosine content of sDNA and pDNA determined by different methods

Different preparations are indicated by the letters in parentheses. sDNA, A–D; pDNA, E–K.

DNA species	Guanine-plus-cytosine content (mol %) determined by:			
	T_m	T.l.c.	Spectral method	Buoyant density
sDNA	36.0 (A)	40.0 (B)	36.8 (B)	40.8 (C)
			38.0 (C)	41.8 (D)
pDNA	36.0 (E)	41.1 (F)	38.5 (H)	37.7 (J)
		38.8 (G)	38.3 (I)	38.8 (K)

Fig. 1. CsCl-density-gradient centrifugation of purified mosquito DNA species

U.v.-absorption photographs of the gradients were taken and the photographs were traced with a microdensitometer. The reference band on the right is *M. lysodeikticus* DNA, density 1.727 g/cm³. The calculated buoyant density of each peak is given. (a) sDNA; (b) pDNA; (c) mixture of sDNA and pDNA.

Physical-chemical characterization of sDNA and pDNA

The base compositions of sDNA and pDNA were determined by the use of four different methods: t.l.c., isopycnic centrifugation, thermal-transition spectra and u.v. absorbance. The results, which are collected in Table 1, show that the average value for the base composition of sDNA was 38.9mol% guanine-plus-cytosine, and for pDNA, 38.5. The buoyant densities of sDNA and pDNA were 1.700 and 1.697 g/cm³ respectively (Fig. 1). The mixture of the two DNA species showed a single band at 1.698 g/cm³. The T_m value for both DNA species was 84°C with approximately 40% hyperchromicity (Fig. 2), and the curves resembled those for double-stranded DNA species.

Fig. 2. Thermal-denaturation profiles of purified mosquito DNA species

The absorbance at each temperature, relative to the absorbance of the native material at 20°C, is plotted as a function of temperature. The T_m value of both DNA species was 84°C. ●, sDNA; ○, pDNA.

Newly-synthesized DNA

Identification of radioactivity in DNA and thymine. Initial experiments were to determine that thymidine was incorporated into larval sDNA and that the radioactive label was in thymine.

Portions of the supernatant fraction from ^{14}C -labelled larvae treated with DNAase or RNAase, and also an untreated sample, were fractionated on a column of Sephadex G-50. The elution profiles (Fig. 3) show that the radioactivity observed in the first peak of the untreated sample, which was in the void volume, was absent from the DNAase-treated sample. Some absorbance at 260 nm, which was probably due to RNA, was still present. The radioactivity of the first peak was present in the RNAase-treated sample, but the E_{260} decreased to half its original value. These results demonstrate that the radioactive component of the supernatant fraction of [^{14}C]thymidine-labelled larvae was DNAase-sensitive and RNAase-insensitive and therefore, was due to its presence in DNA.

T.l.c. of hydrolysates of [^{14}C]thymidine-labelled sDNA showed four u.v.-absorbing spots, which corresponded with the R_F values for adenine, cytosine, guanine and thymine. Over 95% of the initial radioactivity was recovered in the thymine spot, indicating that there was no migration of the radioactive label.

In addition, it was necessary to demonstrate that the acid-precipitable radioactivity of the supernatant fraction from pulse-labelled larvae indeed represented sDNA. When samples of isolated ^{14}C -labelled sDNA and the radioactive supernatant fraction were

centrifuged separately to equilibrium in a preparative CsCl gradient, the radioactive peaks of both samples banded at the same density (Fig. 4). This provided additional assurance that the acid-precipitable radioactivity from the supernatant fractions of pulse-labelled larvae was sDNA.

Size of DNA species *de novo*. The size of isolated DNA species from pulse-labelled larvae was examined by sucrose-gradient centrifugation. After a 2h pulse most of the labelled DNA has a sedimentation coefficient of about 8S in neutral gradients, but sedimented in a broad, heterodisperse zone from 10–19S after a 4h pulse (Fig. 5). Under alkaline conditions the peak fraction of labelled DNA had a sedimentation coefficient of around 8S after pulses up to 4h duration, which indicated that the larger segments of DNA found at 4h were not covalently linked (Fig. 6). Our results also indicated that sDNA is not synthesized by the action of terminal deoxynucleotidyltransferase, which adds deoxyribonucleotides randomly to the ends of DNA strands. This is shown in that after 0.5–6h of pulse, single-stranded, labelled DNA was found only in the 8S band and not in the 19–22S region of larger DNA.

When larvae were pulsed for 6h in [^{14}C]thymidine and 'chased' with non-radioactive thymidine, the sedimentation coefficients of the labelled DNA in alkaline gradients increased to about 10–15S after 24h, and to 17–31S after 48h (Fig. 7). Since the sedimentation coefficients of pDNA under alkaline conditions ranged from 25 to 30S, these experiments indicated that the labelled, newly-synthesized DNA consisted of small-size fragments, which were later combined to form larger pieces in the size range of bulk DNA (pDNA).

The incorporation of the radioactive label into the acid-insoluble fractions containing sDNA and pDNA was also examined in larvae which were pulse-labelled 6h with [^{14}C]thymidine and 'chased' with non-radioactive thymidine (Fig. 8). Although the total radioactivity remained essentially constant throughout the chase period, the concentration of sDNA decreased with a concurrent increase in pDNA. The acid-soluble pool decreased at the same rate as sDNA.

Discussion

The unique characteristic of mosquito sDNA is its relationship to growth, for it constituted 40% of the total DNA in 3-day-old larvae, the most rapid growth stage, and was barely detectable in 6-day-old larvae, pupae and adults when net growth had ceased (Lang *et al.*, 1965; Lang & Meins, 1966). In addition, Lang & Meins (1966) demonstrated that sDNA has a sedimentation coefficient of 8S and bulk DNA (pDNA) 25S, and that sDNA is not the result of enzymatic degradation. The separation of the two DNA species in different subcellular fractions pro-

Fig. 3. Sephadex G-50 profiles of nuclease-treated supernatant fraction

Portions of the supernatant fraction prepared from homogenates of [^{14}C]thymidine-labelled larvae were applied to a column of Sephadex G-50 and the fractions were analysed for radioactivity and E_{260} . Samples: (a) untreated; (b) DNAase-treated; (c) RNAase-treated. O, Radioactivity; ●, E_{260} .

vided an important technical advantage for their isolation and characterization.

These findings raised the most important question, namely 'What is the metabolic role of sDNA?' Our two approaches to this question were the comparison of the physical and chemical characteristics of isolated sDNA and pDNA and the investigation of newly-synthesized DNA as determined by pulse-labelling.

Isolation procedures were developed that yielded about 50% of the DNA present in the supernatant fraction of larval homogenates and about 90% of the DNA in the particulate fraction. Losses of DNA were less when extraction and deproteinization were done with chloroform/butanol rather than with phenol. The isolated DNA species used for physical characterization studies were free from protein and RNA contamination.

The characterization and pulse-labelling experi-

ments suggest that sDNA may have a precursor role in a discontinuous DNA replicative process. Our results show that sDNA has the same physical and chemical characteristics as pDNA, the only difference being size. Our values of 38.9 and 38.5 mol% guanine-plus-cytosine for sDNA and pDNA are in close agreement with the 37.9% G + C composition of an unspecified DNA sample from mosquito larvae reported by Gumilevskaya & Sisakyan (1961).

According to its thermal denaturation characteristics mosquito sDNA is double-stranded. However, unlike pDNA, its secondary structure varies, for as mild a procedure as gel filtration will decrease the T_m value. The strandedness of newly-synthesized DNA species and its interpretation should be regarded cautiously, for it has been shown to be dependent on the isolation procedure (Habener *et al.*, 1970). Also the spontaneous formation of double strands from single-stranded DNA fragments of bacteriophage

Fig. 4. *CsCl*-density-gradient centrifugation of pulse-labelled supernatant and purified sDNA

(a) Larvae (3 days old) were pulse-labelled for 4 h in 10 ml of medium containing 10 μ Ci of [¹⁴C]thymidine (specific radioactivity 51.8 mCi/mmol). A supernatant fraction was prepared and centrifuged in a preparative *CsCl* gradient. The gradient was fractionated from the bottom (left of graph) and acid-precipitable radioactivity and absorbance of each fraction were determined. The total radioactivity was 1693 c.p.m. (b) Profile of purified sDNA from larvae pulse-labelled as in (a) except that 5 μ Ci of [¹⁴C]thymidine were used. The total radioactivity was 508 c.p.m. ●, Radioactivity; ○, *E*₂₆₀.

Fig. 5. *Neutral-sucrose-gradient centrifugation of pulse-labelled DNA*

Portions of DNA extracted from 3-day-old larvae which had been pulsed for 2 or 4 h in 20 ml of medium containing 20 μ Ci of [¹⁴C]thymidine (specific radioactivity, 51.8 mCi/mmol) were analysed on neutral sucrose gradients. Homogenates in a mixture of equal volumes of SSC medium and redistilled phenol equilibrated with SSC medium were centrifuged at 19000*g* for 10 min, and the upper aqueous phase was removed and saved. The 'gummy' phenol phase was shaken again with a half volume of SSC medium and re-centrifuged. The combined aqueous phases were extracted again with SSC medium/phenol, twice with 2 vol. of chloroform/butanol (24:1, v/v), and then incubated overnight at 37°C with 0.4 mg of RNAase/ml which had been pre-treated by heating at 80°C for 10 min in 0.15 M-NaCl, pH 5.0. The solution was applied to a column (30 cm $\times$ 2.5 cm) of Sepharose 6B and eluted with SSC medium. The first u.v.-absorbing peak, which contained the DNA, was concentrated by pervaporation, portions were sedimented through neutral sucrose gradients, and the radioactivity of the fractions was determined. The position of the haemoglobin marker (4.6S) is indicated by the arrows. (a) 2 h pulse; total radioactivity 4075 c.p.m. (b) 4 h pulse; total radioactivity 1491 c.p.m.

Fig. 6. Alkaline-sucrose-gradient centrifugation of pulse-labelled DNA

Axenic larvae, 3 days old, were pulsed as described in the legend to Fig. 5 for various times and then processed in alkali by a modification of the method of Okazaki *et al.* (1968). Larval homogenates in 0.3M-NaOH/0.01M-EDTA/6% (w/v) sodium *p*-aminosalicylate were incubated at 37°C for 1 h and centrifuged at 3000g for 20 min. Portions of the supernatant fraction were sedimented through alkaline sucrose gradients and the acid-precipitable radioactivity of each fraction was determined. The position of marker, ϕ X174 DNA (22.5S), is indicated by the arrow. ●, 0.5h; ○, 1h; ▲, 2h; △, 4h.

Fig. 7. Alkaline-sucrose-gradient centrifugation of pulse-labelled and pulse-chased DNA

Axenic larvae, 3 days old, were pulsed for 6h as described in the legend to Fig. 5 and then 'chased' for 1 or 2 days with a 1000-fold excess of thymidine. The processing conditions are described in the legend to Fig. 6. ●, 6h pulse, no chase; total radioactivity 9475 c.p.m.; ○, 6h pulse, 1 day chase; total radioactivity 6255 c.p.m.; △, 6h pulse, 2 day chase; total radioactivity 6220 c.p.m.

T-4 has been demonstrated (Barzilai & Thomas 1970).

Differences in the secondary structure of newly-synthesized DNA have been reported with investigators finding it to be double-stranded (Tsukada *et al.*, 1968; Probst *et al.*, 1971; Hershey *et al.*, 1973) or containing both double- and single-stranded characteristics (Habener *et al.*, 1969; Taylor *et al.*, 1973; Gautschi & Clarkson, 1975). Although sDNA appears to be double-stranded when isolated by our procedure, the possible existence of single-stranded regions needs verification.

DNA in pulse-labelled larvae is apparently synthesized initially in small pieces of fairly uniform size which become part of larger structures with increasing pulse time, but which are not covalently linked during the first 4–6h of synthesis. When pulse-labelled DNA is 'chased' in non-radioactive thymidine, the size of the labelled component gradually increases until much of it sediments at about the same rate as pDNA in alkaline gradients. Thus sDNA appears to be covalently joined during the

Fig. 8. Incorporation of [14 C]thymidine into sDNA and pDNA in mosquito larvae

Axenic larvae, 2 days old, were pulsed 6h in 20ml of medium containing 20 μ Ci of [14 C]thymidine (specific radioactivity, 45.5 mCi/mmol) and 'chased' with 20000-fold excess of non-radioactive thymidine. Larvae were sampled and processed at several time-points to determine the acid-precipitable and acid-soluble radioactivities of the different fractions. ●, sDNA; ○, pDNA; △, acid-soluble fraction.

time of the chase to form larger DNA fragments, which finally become pDNA.

These characteristics of sDNA are similar in some respects to those reported for DNA replication intermediates in other eukaryotic organisms and cell types. Although newly-synthesized DNA fragments approximately the same size as sDNA have been found in HeLa cells (Habener *et al.*, 1969), in human heteroploid cells (Nuzzo *et al.*, 1970), and in Chinese-hamster cells (Schandl & Taylor, 1969), a distinct variability in the sizes has been reported by different investigators, and, with increasing pulse time, a range of sizes was found that may represent different degrees of replication (Taylor *et al.*, 1973; Gautschi & Clarkson, 1975).

In other pulse-chase experiments that measured the incorporation of [¹⁴C]thymidine into acid-insoluble material, a similar shift of radioactivity from the sDNA and acid-soluble fractions to pDNA was observed. Although these data do not eliminate the possibility that there was also incorporation of thymidine directly into pDNA, the sucrose-gradient experiments showed that the radioactive label was initially incorporated only into the small-size fragments.

The reason for the occurrence of sDNA in the supernatant fraction from ultracentrifugation of larval homogenates is not known. A mitochondrial origin of sDNA has been rejected because it is of small size, unlike mitochondrial DNA, and because it is found in supernatant fractions which are free of mitochondria. The similar base compositions of sDNA and pDNA would not be expected in satellite or mitochondrial DNA species, or in gene-amplification systems (Brown & Dawid, 1968). Radioautographs of sections from larvae pulsed in radioactive thymidine show that the label is localized only over nuclei (P. C. Kao & C. A. Lang, unpublished work).

The possibility that sDNA is a product of endogenous DNAase action on pDNA has been carefully considered and appears unlikely. Experimental evidence against this possibility include the quantitative recovery of calf thymus DNA added to larvae which were processed and stored in the cold for several hours and the failure to alter the amount of sDNA when live larvae were homogenized in media containing citrate or EDTA, which chelate Mg²⁺ essential for DNAase activity (Lang & Meins, 1966).

The high concentration of sDNA during the exponential growth phase of mosquito larvae suggests that its metabolic role is not trivial. The most rapid increase in total DNA content also occurs during the first 4 days of larval life (Lang *et al.*, 1965). These experiments indicate that sDNA is involved in the replication of DNA and may represent an intermediate form in the overall process. Moreover the results also suggest that there are at least two major

steps in mosquito DNA replication, namely the synthesis of small fragments (sDNA) and the joining of these fragments to form pDNA. This was shown by an accumulation of small-size DNA during a 0.5–6 h pulse period which preceded the formation of larger DNA.

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