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Characterization of the DNA and Chromosonal Analysis of Embryonic *Dermacentor variabilis* (SAY) Cells in Culture

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CHARACTERIZATION OF THE DNA AND CHROMOSOMAL ANALYSIS OF EMBRYONIC *Dermacentor variabilis* (SAY) CELLS IN CULTURE

by

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ABSTRACT

CHARACTERIZATION OF THE DNA AND CHROMOSOMAL ANALYSIS OF EMBRYONIC *Dermacentor variabilis* (SAY) CELLS IN CULTURE

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Cultured embryonic *Dermacentor variabilis* cells were examined by spectrophotometric techniques to determine total cellular DNA concentration and guanine-cytosine content of the DNA and by differential staining procedures to define the karyotype further. The cells were found to have a mean DNA content of 16.98 pg and a DNA guanine-cytosine content of 49%. Chromosome banding attempts resulted in the specific characterization of four chromosomes and differential grouping of the seven remaining chromosomes. A previously unreported submetacentric chromosome was identified.

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Introduction

Ticks are second in importance to mosquitoes as vectors of pathogens to man and are the most important vector of pathogens to vertebrate animals (Yunker & Meibos, 1979). Understanding the methods by which these pathogens are harbored by the ticks and are transmitted from one tick generation or developmental stage to the next can lead to an understanding of their epidemiology. A number of these tick-borne pathogens are viruses which may be transmitted transstadially (e.g., Varma & Smith, 1962) or both transstadially and transovarially (e.g., Naumov et al., 1980a,b). Although several mechanisms for this viral persistence in ticks have been proposed, the precise mechanisms remain unknown (Varma & Smith, 1972; Preble & Younger, 1975; Andzhaparidze et al., 1978). Proposed vector-virus interrelationships and vector competence are reliable only when vector species are sharply defined. It is, therefore, desirable to establish a foundation of cytological and molecular knowledge which will aid in species definition and, by so doing, provide some insight into evolutionary relationships (Hinegardener, 1976; Koulischer, 1973).

Viral persistence in cells involves the chemical interaction of viral and host species' nucleic acids

(Fenner et al., 1974). Therefore, it is essential to utilize existing techniques to investigate the nucleic acids of host ticks as well as tick-borne viruses to understand tick-virus association.

Very little is known of the molecular genetics of ticks or tick-borne viruses. Studies of the transfection ability of DNA isolated from cells infected with arboviruses indicated a proviral mechanism for Bahnja virus and tick-borne encephalitis virus (TBEV) in host cells (Gaidamovich et al., 1976; Barinskii et al., 1978). A proviral mechanism was also suggested by studies involving the hybridization of DNA from TBEV infected cells with TBEV RNA and separately with cDNA synthesized from a TBEV RNA template (Zhdanov et al., 1974; Andzhaparidze et al., 1978). Also, Knudson (1981) studied the genome of Colorado tick fever virus and found twelve segments of double-stranded RNA. Beyond these, nothing is known at the molecular level of DNA from tick cells or of the viruses tick cells harbor.

More information is available on the cytogenetics of ticks. Oliver (1977) reviewed the sex determining mechanisms and/or karyotype data such as chromosome numbers, lengths, and types for 24 argasid and 70 ixodid tick species. However, all of these studies relied on uniform chromosome staining and no successful tick chromosome banding attempts have been reported in the literature.

The ixodid tick species Dermacentor variabilis is important as a vector for pathogens causing Rocky Mountain spotted fever, tularemia, Colorado tick fever, St. Louis encephalitis and other diseases of man and animals (Hoogstraal, 1966; Arthur, 1962). The objectives of the current study are to determine the cellular DNA content, percent guanine-cytosine of the cellular DNA, and the chromosome banding pattern for this medically and veterinarily important tick species.

Materials and Methods

Ticks

Ticks used in this study were laboratory-reared Dermacentor variabilis (Say) from a stock obtained on a farm near Montpelier, Hanover County, Virginia. Ticks were maintained in an Aminco Climate Lab^{R} environmental chamber at 27±0.5C and 90±2.0% RH. They were fed and mated on albino rats, Rattus norvegicus, and held in individual, sterile, cotton-stoppered shell vials during egg laying.

Cell Lines

Continuous cell line cultures (CC) were obtained from Dr. Conrad Yunker, Rocky Mountain Laboratory, National Institutes of Health, Hamilton, Montana (RML) and were derived at RML from embryonic cells of laboratory-reared D. variabilis. These cultures were

maintained in Yunker-Meibos medium (Yunker & Meibos, 1979) in a 37C incubator.

Primary cultures (PC) were developed according to the technique described by Yunker and Meibos (1979) from embryonic tissue 8-12 days post-initiation of oviposition.

DNA Analyses

Total Cellular DNA Concentrations

Cells were harvested from confluent CC cultures using a rubber policeman. Cell counts were made with a Levy Chamber Hemacytometer, Model 500 (Hausser Scientific, Township Line, Blue Bell, Pa.) and the total number of cells in each flask was estimated from these counts. Tick cell homogenates were prepared by grinding the cells in a phosphate-saline buffer (0.05M $NaPO_4$, 2.0M NaCl, pH 7.4) using a Potter-Elvehjem tissue grinder with a teflon pestle (Wheaton Scientific, Millville, N.J.). Total cellular DNA amount was determined by using the fluorescent stain Hoechst 33258 (Bisbenzimide, lot number 98C-0182), Sigma Chemical Corporation, St. Louis, Mo.) and a Perkin-Elmer Fluorescent Spectrophotometer (Model 204) with a Perkin-Elmer Xenon Power Supply, Model 150 (Perkin-Elmer Corporation, Norwalk, Conn.) according to the procedure of Labarca and Paigen (1980) for DNA estimates using whole cell homogenates. Standards were made using

isolated salmon sperm DNA obtained from Dr. Thomas Sitz, Department of Chemical Sciences, Old Dominion University, Norfolk, Virginia. Non-fluorescent spectrophotometric DNA concentration measurements were made on the standards using a Beckman Spectrophotometer, Model 26 (Beckman Instruments, Inc., Fullerton, Calif.). The absorbance read at 260nm for each standard was multiplied by 50 [the native DNA concentration (μg/ml) per A_{260} unit as reported by Clark and Switzer (1977)] to give the total cellular DNA concentration in μg/ml of solution.

The total cellular DNA concentration in the D. variabilis cell homogenates was read from a linear regression plot of the fluorescence vs. DNA concentration of the salmon sperm standards. Equation 1 is the computer equation used for the linear regression line calculation (Whitsitt et al., 1981). In equation 1, N is the number of standards, x is the DNA concentration (μg/ml) of a given standard, y is the fluorescence corresponding to x, x' is any value for the DNA concentration and y' is the fluorescence corresponding to x'. A correlation coefficient calculation was preprogrammed in the computer used for the linear regression analysis and was performed automatically with the linear regression analysis data. The DNA concentration/ml of homogenate was divided by the number of cells/ml of homogenate to give the D. variabilis DNA content/cell.

$$y' = \frac{\frac{\Sigma x \Sigma y}{N} - \Sigma xy}{\frac{(\Sigma x)^2}{N} - \Sigma x^2}(x') + \frac{\Sigma y}{N} - \frac{\frac{\Sigma x \Sigma y}{N} - \Sigma xy}{\frac{(\Sigma x)^2}{N} - \Sigma x^2}\left(\frac{\Sigma x}{N}\right) \quad (1)$$

GC Content

DNA was extracted from cells of confluent CC cultures following the DNA isolation procedure I of Butterworth (1976) and stored at 4C in 0.1 x saline sodium citrate (SSC) pH 7.0±0.3 until used. The DNA-GC content was estimated by melting point determination of the isolated DNA (Marmur & Doty, 1962). To establish the appropriate sodium ion concentration, samples were dialyzed in 1 x SSC (pH 7.0±0.3) using Spectrapor Membrane Tubing (Spectrum Medical Industries, Inc., Los Angeles, Calif.) with a dry cylinder diameter of 11.5 mm and a molecular weight cutoff of 3,500 daltons. Prior to dialysis, tubing and tubing clamps were boiled sequentially in 5% $NaHCO_3$, deionized H_2O, 10 mM EDTA and twice in deionized H_2O to destroy any DNAse activity. One ml volumes of isolated and salmon sperm DNA solutions were placed in separate dialysis bags, immersed in 1 ℓ of sterile 1 x SSC at 4C and stirred slowly for 24 hr with the SSC replaced at 6 hr intervals.

Tm Determination

The dialysates of both the D. variabilis and salmon sperm DNA samples were diluted with 1 x SSC to a

concentration of about 75 µg/3 ml. These were placed in 3 ml quartz cuvettes and degassed by bubbling helium through the DNA solutions for 3 min to prevent bubble formation while heating. To reduce the chance of cuvette explosion due to thermal expansion of trapped gases during the Tm determination, the cuvettes were capped immediately after the helium treatment. To obtain DNA thermal melt data for Tm determination, the absorbance at 260 nm was determined for each sample in a Cary 219 Spectrophotometer (Varian Instrument Division, Palo Alto, Calif.) where the temperature of the sample could be raised by circulating hot water through the coupled turrets accessory. The water was heated by means of a Neslab Exacal 100 heater with a Neslab ETP-3 temperature programmer (Neslab Instruments Inc., Portsmouth, N.H.) to regulate the rate of heating. The turret temperature monitor was placed in a photocell cuvette containing glycerol to check the true temperature within the photocell chambers. This was necessary since there is a 10C drop in temperature between the heater and the photocells.

To determine the Tm of the DNA, the optical density was read at 260 nm as the temperature was increased from 25 to 98C at a rate of 0.8C/min. A recorder attached to the Cary 219 Spectrophotometer recorded A_{260} with increasing temperature. The reading was controlled by a Varian Timer Accessory (Model 954000)

with a range setting of 0.2, record time of 15 sec/channel and a cycle time of 1 min. The range setting was changed from 0.2 to 0.5 at a temperature of approximately 87C in the first DNA thermal melt to fit the denaturation curve on the recorder printout. The gain was set at 0.26 giving a spectral band width of 0.6. The period was set at 1.

DNA thermal melt temperatures and their corresponding absorption values were taken every 0.5C from the hyperchromic regions of the Cary 219 recorder printouts of the DNA thermal melts. The absorption values were corrected for the thermal expansion of water according to Felsenfeld (1968) using equation 2 where A^{c}_{260} is the thermal expansion corrected absorption value, A_{260} is the observed absorption value, V_2 is the specific volume of water at the measurement temperature and V_1 is the specific volume of water at 4C. The values for the specific volume of water at various temperatures were taken from Lange (1956) or interpolated from those values.

$$A^{c}_{260} = A_{260} \left(\frac{V_2}{V_1}\right) \qquad (2)$$

The temperature corresponding to the peak of a plot of the $\Delta A^{c}_{260}/\Delta 1C$ vs pTC (where pTC is the temperature corresponding to the midpoint of the $\Delta 1C$ temperature rise) was designated as the Tm (Van et al., 1976). The GC

content was calculated from equation 3 where Tm is in degrees celsius and (G-C) refers to the mole percentage of guanine plus cytosine (Marmur & Doty, 1962).

$$(G-C) = T_m - 69.3/0.41 \quad (3)$$

Chromosomal Analysis

To arrest the cell cultures (either PC or CC) in metaphase of mitosis, colcemid (Gibco Diagnostics, Grand Island, N.Y.) was added to the cultures at a concentration of 0.15 µg/ml of medium and the flasks were incubated for 2.5 hr at 37C. The cells were then detached with a rubber policeman, pelleted, resuspended in 8 ml of 0.075 M KCl for 20 min at room temperature and centrifuged at 1000 rpm. They were fixed overnight in 10 ml of methanol:acetic acid (3:1 v/v) with 1 change of the fixative after 5 min. Thereafter, the cells were centrifuged at 1000 rpm and resuspended in 0.5 ml fixative. All centrifugations were performed in an International clinical centrifuge (International Equipment Co., Needham Hts., Mass.).

Slides for chromosomal analysis were prepared as follows: clean slides were dipped in distilled H_2O and six drops of cell suspension dropped on each from a distance of 6 to 24 inches. The slides were air dried at room temperature or heated with a flame or slide warmer to increase the drying rate.

Aliquots of a concentrated quinacrine mustard dihydrochloride (Sigma) stock solution were added to a coplin jar of distilled H_2O until the staining solution was a bright yellow (i.e., at a concentration of roughly 5 ml quinacrine mustard/100 ml H_2O). Prepared slides (of either PC or CC cells) were dipped in the quinacrine staining solution for 2 to 3 min, rinsed in tap water, dipped in phosphate buffer (0.02 M citric acid, 0.11 M sodium phosphate dibasic, pH 5.6) and fitted with a cover glass over phosphate buffer.

Photomicrographs of chromosome spreads were taken with an Olympus PM-10-A photomicrographic system (Olympus Optical Co. Ltd., Tokyo, Japan) on a Zeiss research microscope (Zeiss Microscope Co., Oberkochen, West Germany) employing a 50-watt high pressure mercury lamp power supply. Kodak technical pan film 2415 (Eastman Kodak Co., Rochester, N.Y.) was used for all photographs and developed in Kodak general purpose developer. Figures were printed on Kodak Ektamatic Sc photographic paper F using a Besseler 45 MXII enlarger (Besseler Photo Marketing Co., Inc., Florham Park, N.J.) and the prints processed in a Kodak Ektamatic processor.

A second procedure for fluorescent microscopy was attempted to supplement and confirm the results of the quinacrine mustard staining. CC cultures were incubated 2-days post-subculturing in Yunker-Meibos

medium containing 1 x 10^{-6}M methyltrexate (Sigma) for 17 hr at 37C. The medium was poured off and the cells washed twice with medium not containing methyltrexate. Medium containing bromodeoxyuridine (BrdU) (Sigma) at a concentration of 60 µg/ml was added to the cultures and they were incubated an additional 7.5 hr at 37C and harvested as previously described. Prepared slides were stained for 5 min in acridine orange (Sigma) (5 mg acridine orange in 100 ml of 0.025 M KH_2PO_4 buffer, pH 6.8), rinsed 2x in phosphate buffer for 1 min each, and fitted with a cover glass over buffer. Chromosome spreads were analyzed by fluorescent photomicrography as described previously.

Results

DNA Analyses

Total Cellular DNA Concentration

Total cellular DNA concentrations for D. variabilis cell homogenates and DNA amounts in salmon sperm standards are shown in Table 1. The linear regression line used to estimate the total amount of DNA in the D. variabilis cell homogenates from their fluorescence is shown in Figure 1. The linear regression line had a correlation coefficient of 0.99 when compared to the experimental data.

Table 1. DNA concentration of *Dermacentor variabilis* cell homogenates and salmon sperm standards.

Sample[1]	Fluorescence Units	A_{260}	µgDNA/ml	cells/ml
D.v. A	49.7	NA	9.65[2]	5.47×10^5
D.v. B	49.8	NA	9.68[2]	5.93×10^5
ss A	29.7	0.061	3.05	NA
ss B	32.9	0.076	3.80	NA
ss C	35.8	0.086	4.30	NA
ss D	38.6	0.106	5.30	NA
ss E	40.2	0.138	6.90	NA
ss F	44.5	0.158	7.90	NA
ss G	52.2	0.208	10.40	NA

[1]D.v. = *Dermacentor variabilis* embryonic cell homogenates. ss = salmon sperm standard solutions.

[2]Derived by linear regression (see text).

NA = not applicable

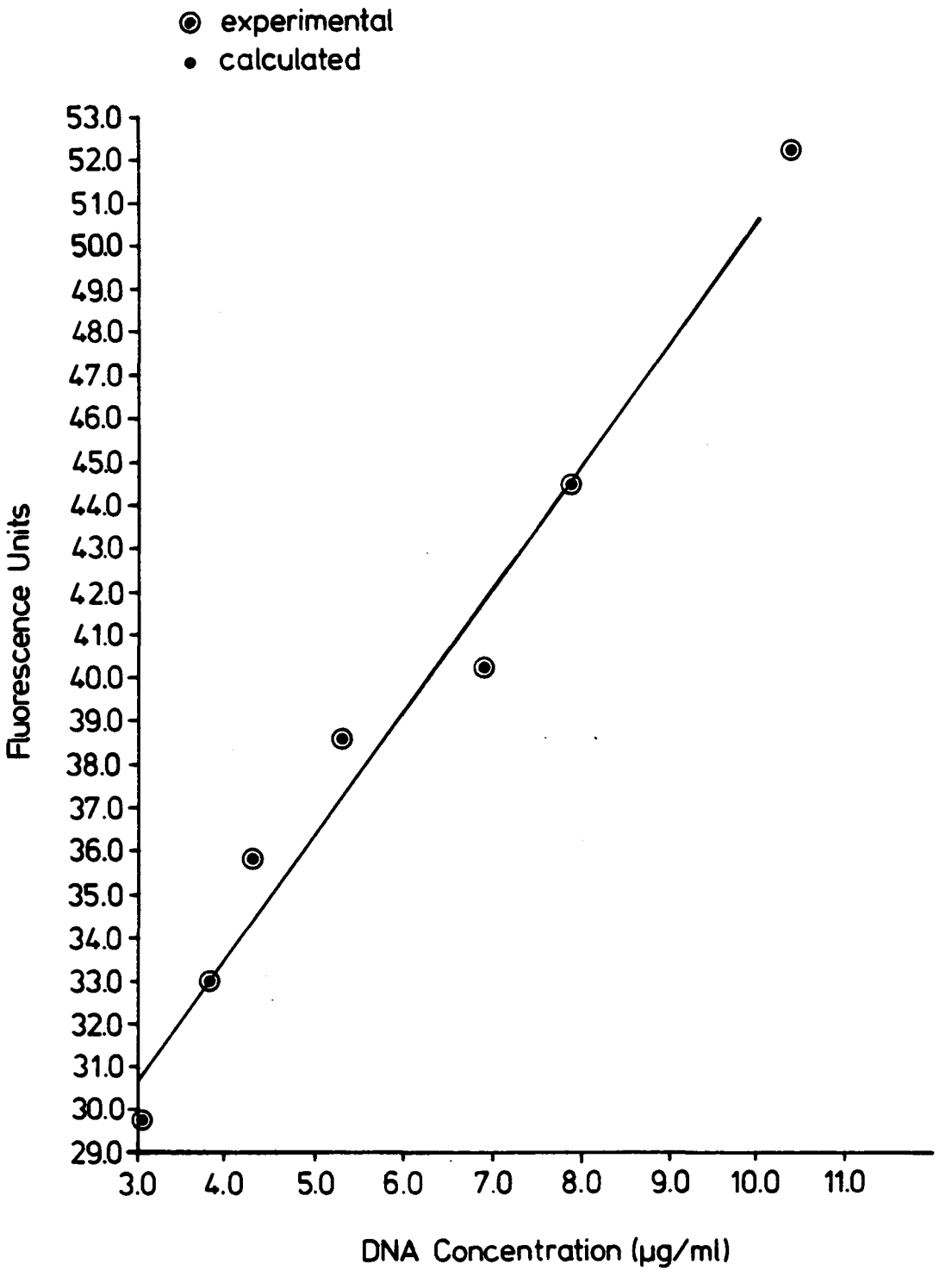

Fig. 1 Linear regression analysis of salmon sperm DNA standards.

Diploid D. variabilis cells in culture had values of 17.64 pg and 16.32 pg for two samples analyzed ($\overline{M}$ = 16.98 pg).

GC Content

Plots of the A^{C}_{260} vs temperature for the D. variabilis and salmon sperm DNA thermal melt runs I and II and the averaged thermal melt data are shown in Figure 2. To give a sufficiently large data base, the average thermal melt data was used in Tm determinations (Figure 3).

Average thermal melt data indicated Tm values for salmon sperm DNA and D. variabilis DNA of 86.5C and 88.5C respectively. The former temperature was 1C lower than the comparable value reported for salmon sperm DNA by Marmur and Doty (1962). This variation was probably caused by uncontrollable factors (e.g., small saline solution differences, instrument-induced variables, etc.); therefore, 1C was added to the D. variabilis Tm before comparing it with those figures reported for other species. The adjusted Tm value for D. variabilis DNA was 89.5C which corresponds to a guanine-cytosine content of 49%.

Chromosomal Analysis

Figure 4 shows a karyotypic display of a typical male metaphase plate and Figure 5 a karyotypic display of

D.V. = Dermacentor variabilis
S.S. = Salmon sperm
I = First run
II = Second run

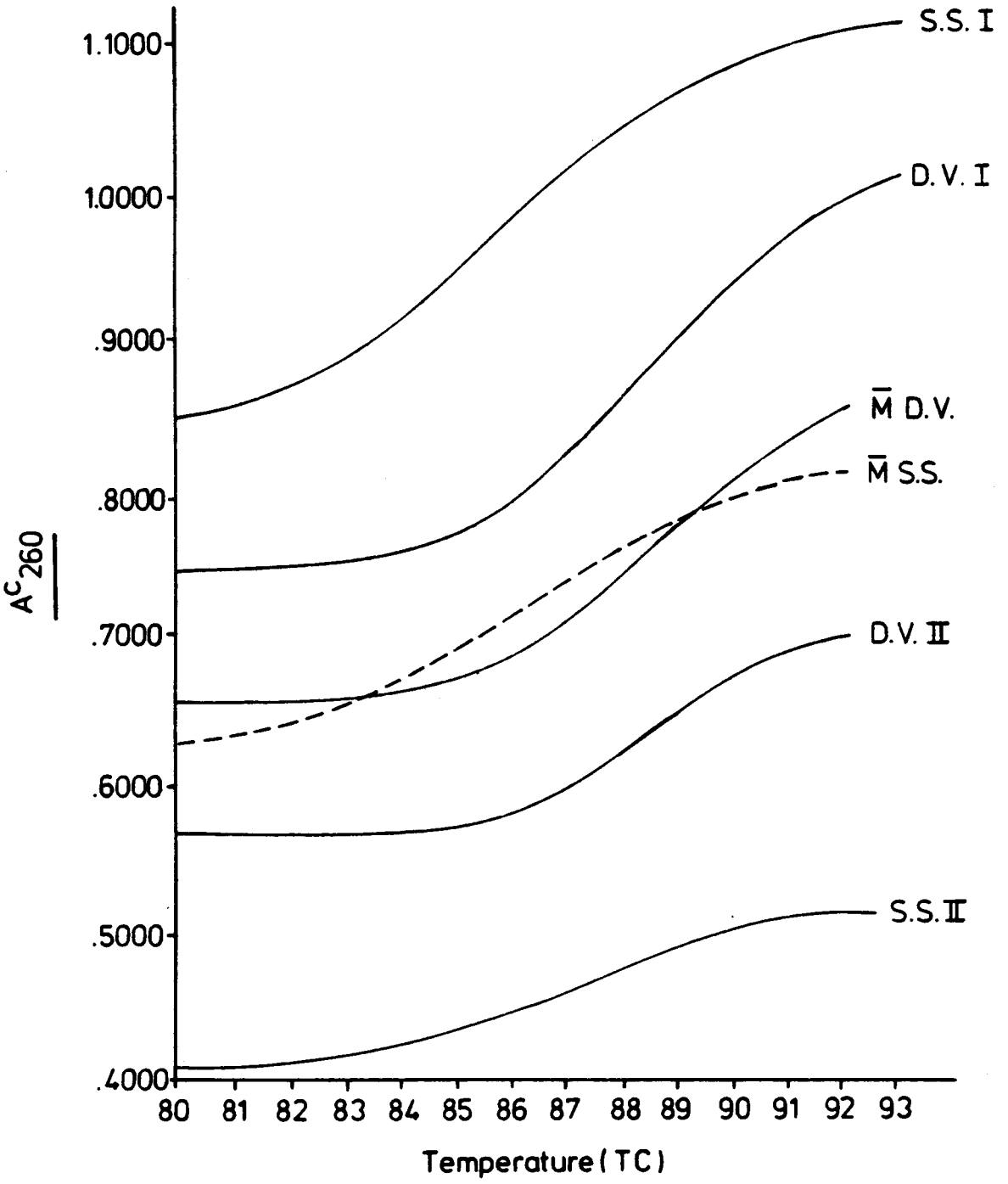

Fig. 2 Thermal melt data for Dermacentor variabilis cultured cell and salmon sperm DNA's.

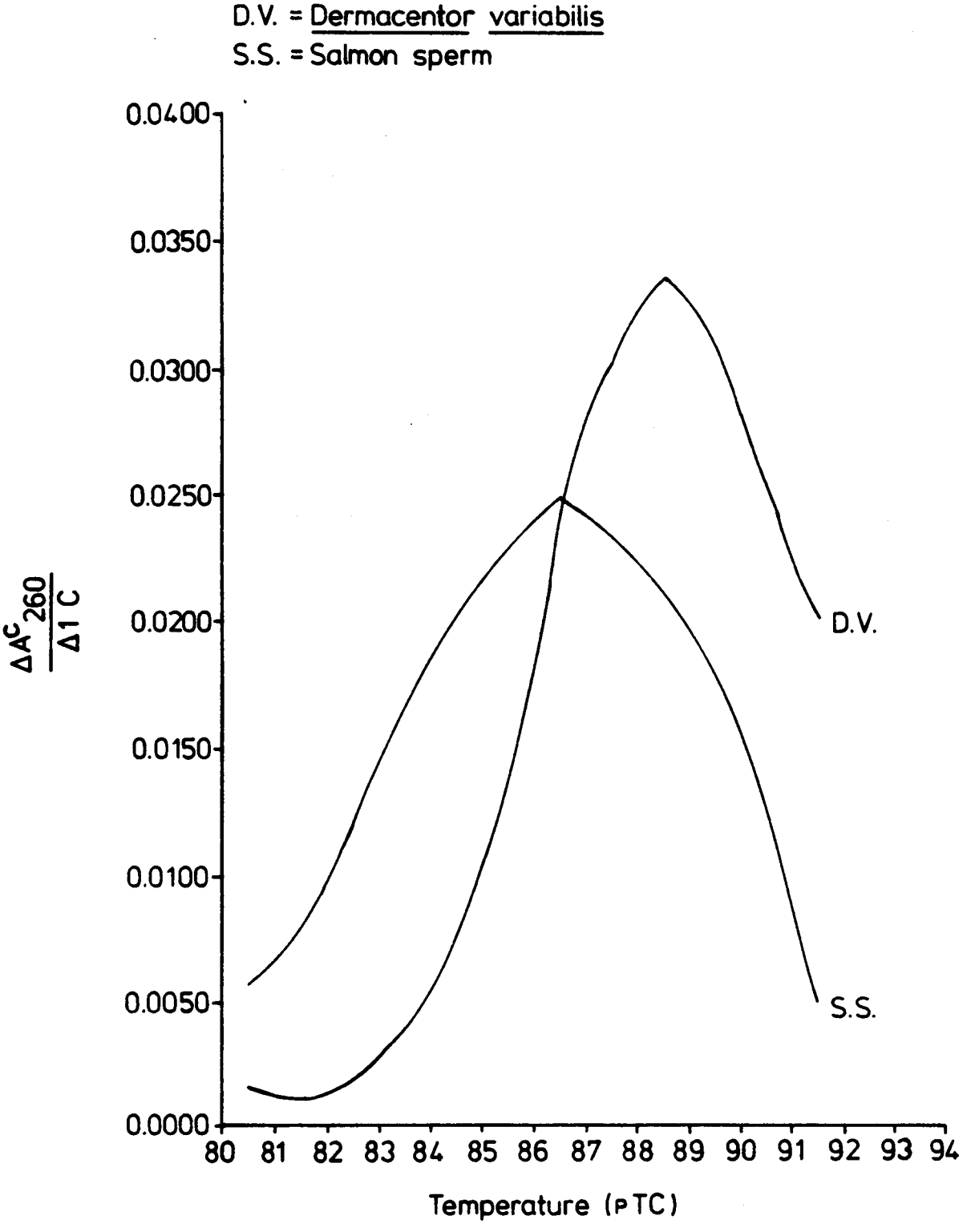

Fig. 3 Average thermal melt data for Dermacentor variabilis and salmon sperm DNA's.

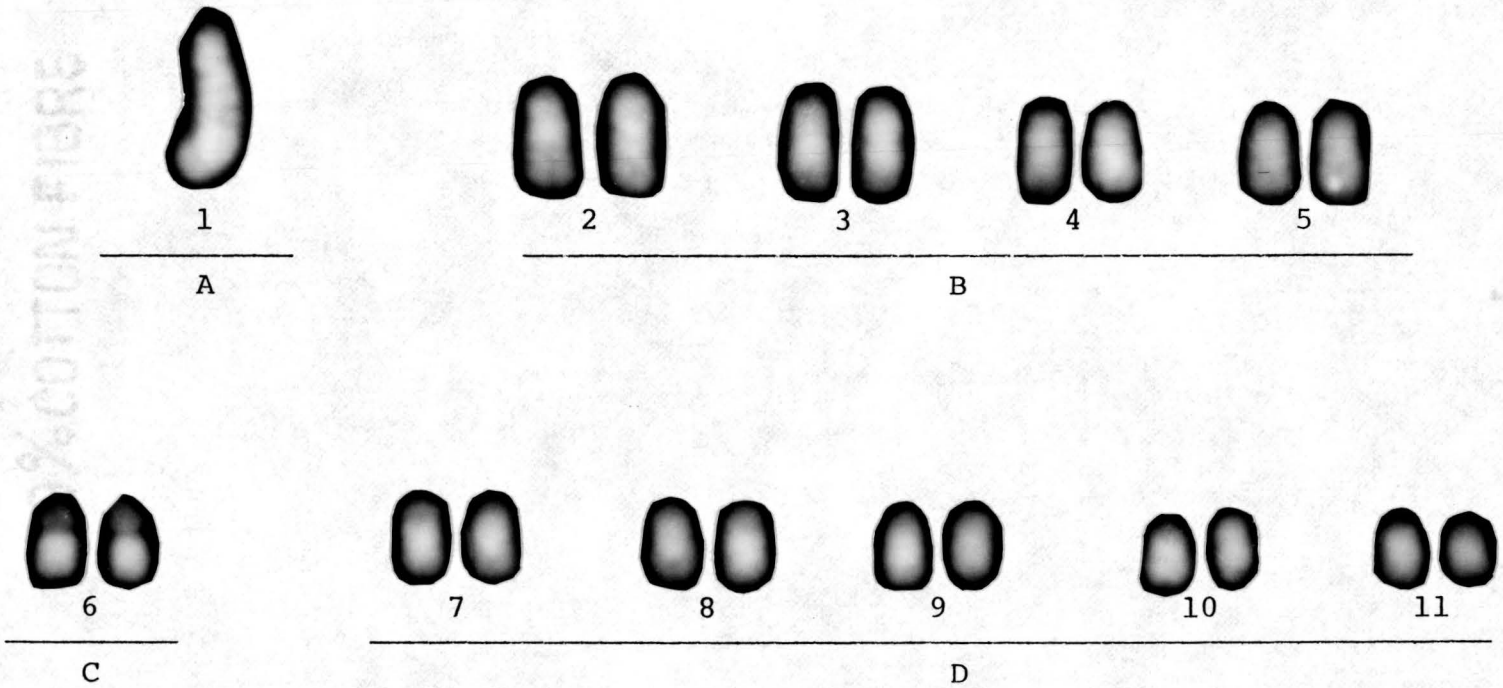

Fig. 4. Typical karyotyped Dermacentor variabilis PC cell male metaphase plate following quinacrine mustard treatment.

a typical female metaphase plate of the PC cells after quinacrine mustard treatment. Figures 6 and 7 show typical male and female chromosome spreads from which Figures 4 and 5, respectively, were derived.

The chromosomes were arranged into four groups on the basis of length and centromere location. It was possible to assign the chromosomes to appropriate groups by size alone except for a single submetacentric chromosome which was identified by centromere location. The largest chromosomes were given the lowest letter designation.

Group A consists of the X chromosome (pair) which is the largest and is acrocentric.

Group B consists of chromosomes two through five which are medium length acrocentrics. The presence of a bright band one-third of the way down the arm of chromosome two and a bright band in the middle of chromosome three aid in their specific identification. Chromosomes four and five cannot be distinguished by their banding patterns.

Group C consists of a submetacentric chromosome pair characterized by a dimly fluorescent short arm and a brightly fluorescent long arm.

Group D consists of chromosomes seven through eleven which are small acrocentrics, none of which present a distinguishing banding pattern.

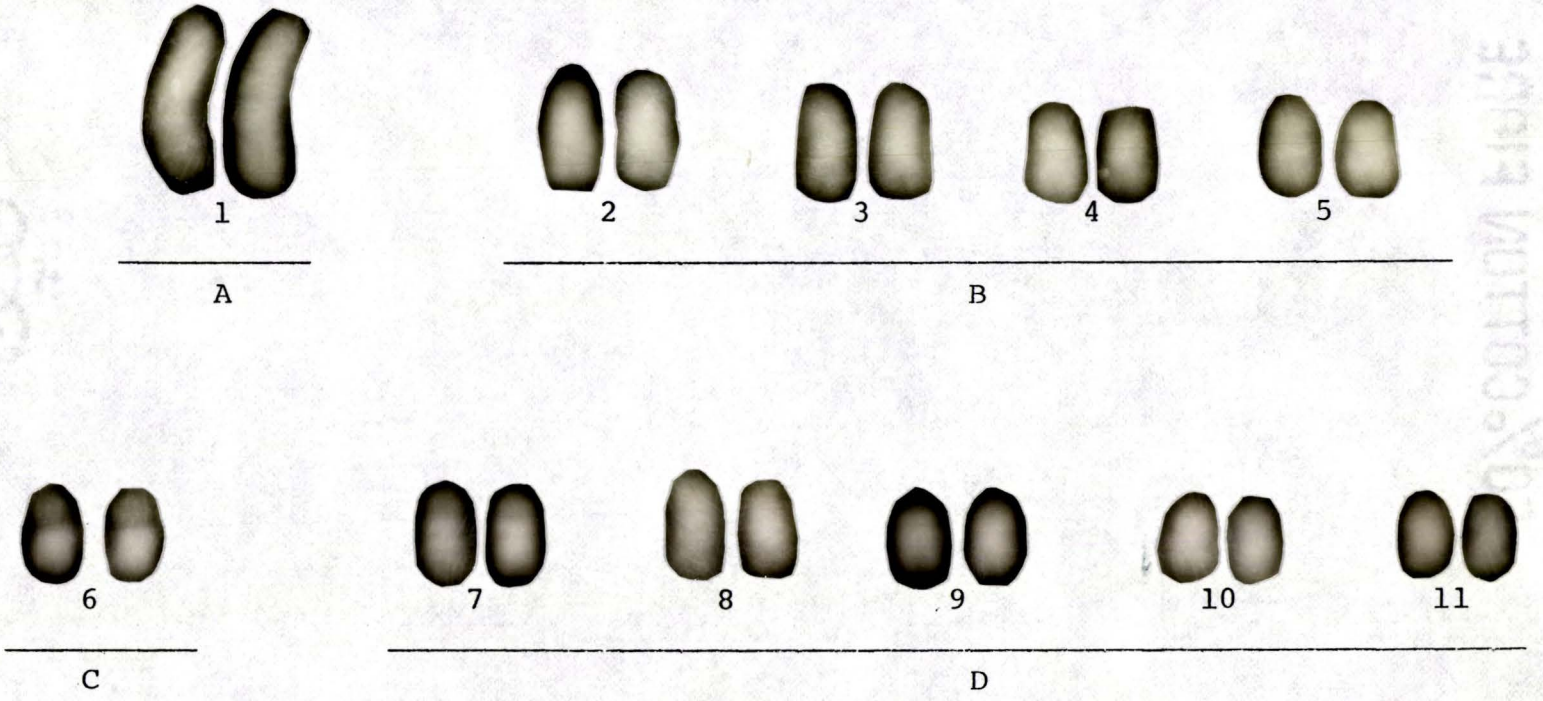

Fig. 5. Typical karyotyped Dermacentor variabilis PC cell female metaphase plate following quinacrine mustard treatment.

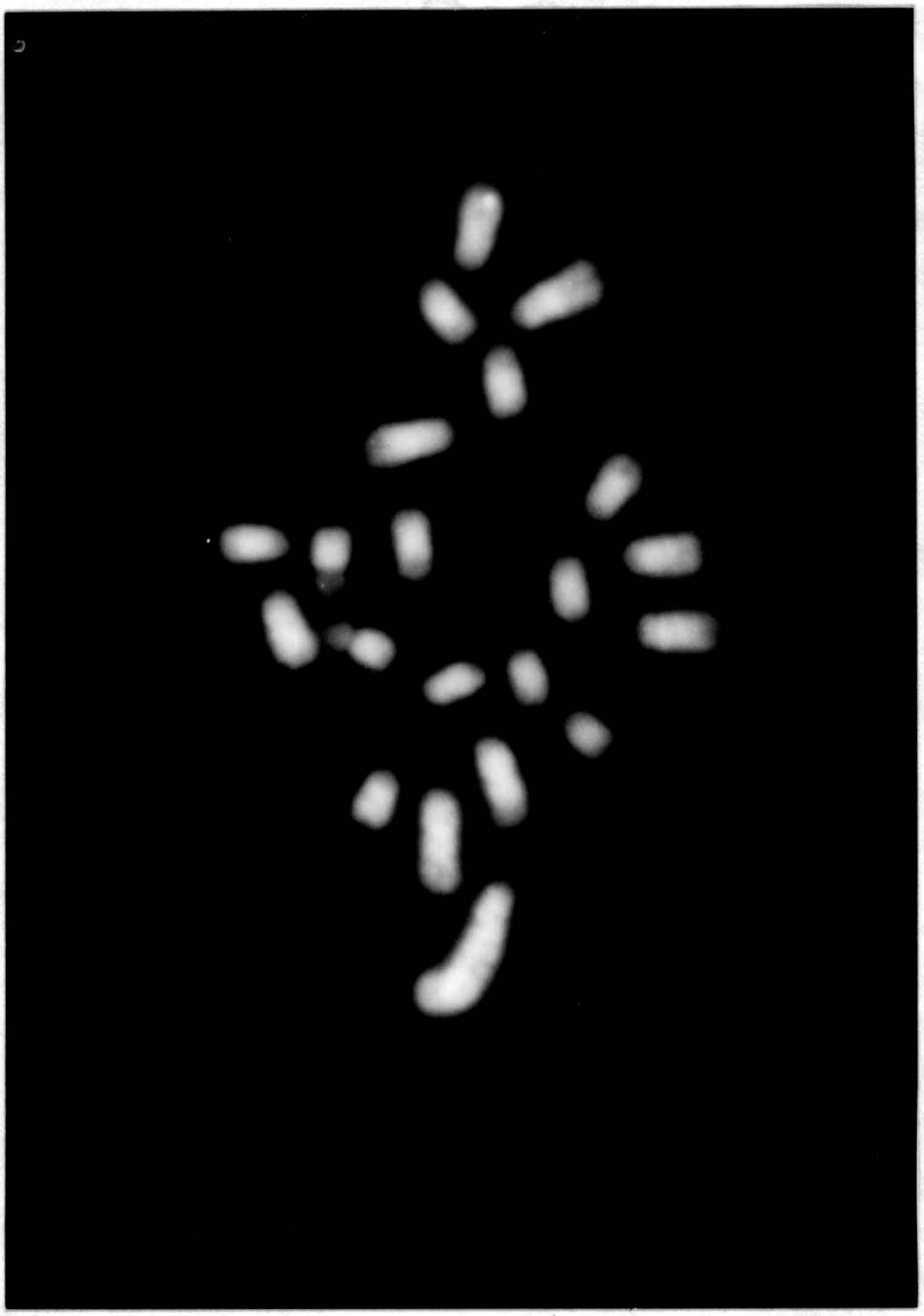

Fig. 6. Typical Dermacentor variabilis PC cell male metaphase plate following quinacrine mustard treatment. x3,750

Fig. 7. Typical Dermacentor variabilis PC cell female metaphase plate following quinacrine mustard treatment. x3,750

Figure 8 Shows a typical metaphase plate from CC cells after BrdU-acridine orange treatment. The appearance of submetacentric chromosomes near the center and in the lower left of the spread (see arrows) confirms the presence of the submetacentric acrosome in D. variabilis CC cultures. Chromosomes from the CC cells were unremarkable after quinacrine mustard treatment.

Discussion

DNA Analyses

Total Cellular DNA Concentration

The total cellular DNA content analysis reported in this study fits the general trend of an increase in DNA content as you follow the phylogenetic trend from the lower invertebrates to the higher invertebrates (Mirsky & Ris, 1951; Hinegardener, 1976). Although the total cellular DNA content reported here is roughly three times that of the only arachnid value reported (Britten & Davidson, 1971), a range up to two orders of magnitude for cellular DNA contents within the same class of animals is known. For example, insect values range from 0.085 pg for the dipteran species Drosophila melanogaster to 7.5 pg for the grasshopper Melanoplus differentialis and the values for amphibians range from 1.05 pg in the frog Scaphiopus holbrookii holbrookii to 95 pg in the Congo eel Amphiuma means (Sparrow et al., 1972).

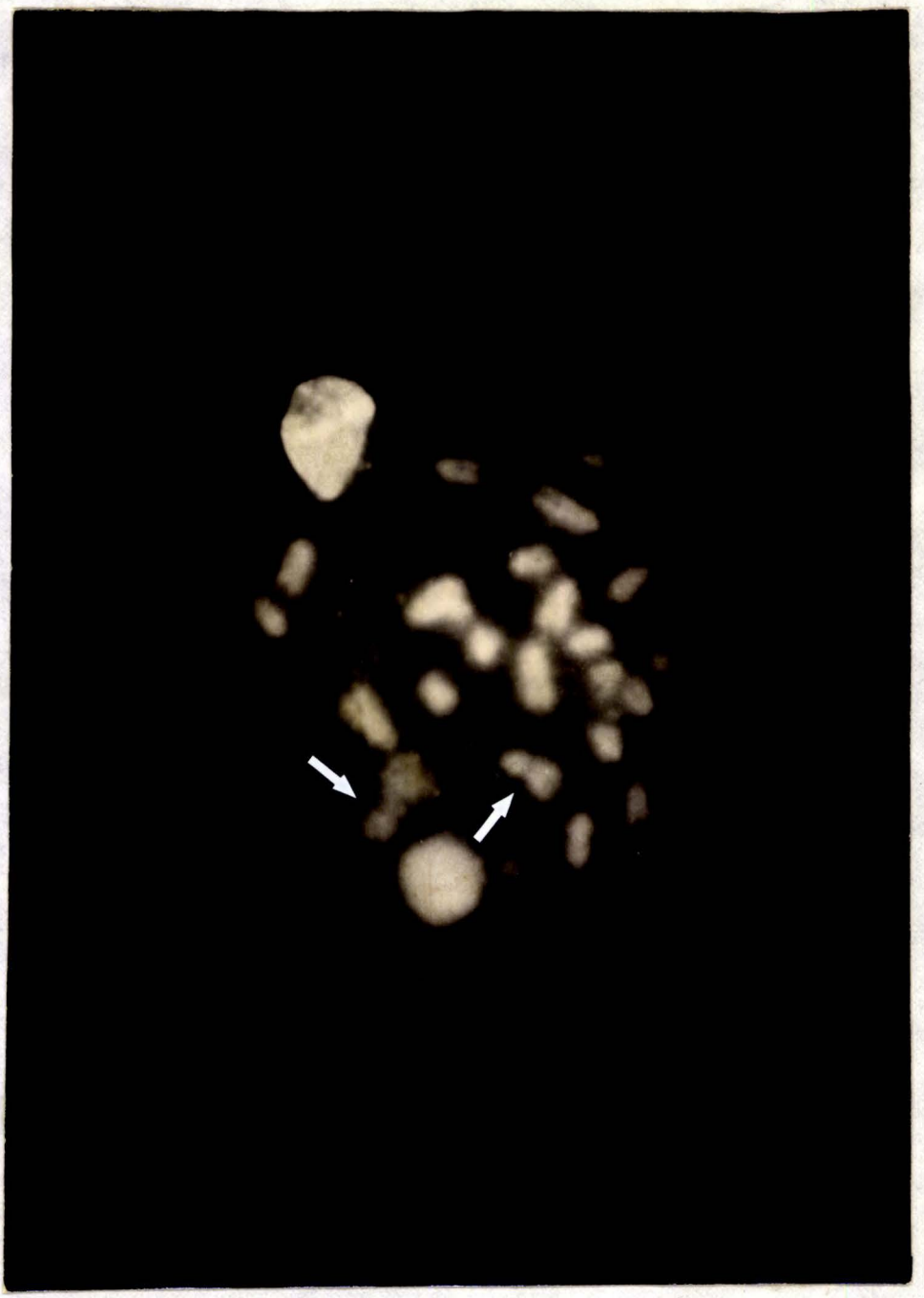

Fig. 8. Typical Dermacentor variabilis CC cell metaphase plate following BrdU-acridine orange treatment. x3,750

Considering all arthropods, a cellular DNA content of 8.49 pg is within the reported cellular DNA content range (0.085 pg to 15.8 pg per haploid genome). However, prior to this study the only arthropod values reported above 7.5 pg were those of some crustacean species (Hinegardener, 1976).

There is an accepted evolutionary trend that animals considered to represent primitive or ancient and relatively slowly evolving lineages (that are not highly specialized) are often found at the higher end of the range of cellular DNA content for their particular taxon (Hinegardener, 1976). D. variabilis resides in the super-family Ixodoidea, which arose in the late Paleozoic or early Mesozoic era (Savory, 1977; Hoogstraal, 1978). The genus Dermacentor probably did not appear until the Tertiary period (Hoogstraal, 1978). D. variabilis has adapted to extremes in environment that restrict the range of other species (Sonenshine, 1979). These factors suggest a primitive and relatively slowly evolving lineage which is not highly specialized and in which high cellular DNA contents are more frequent.

Although as ectoparasites the ticks are necessarily considered specialized, D. variabilis is largely unspecialized with respect to mammalian host. Smith et al. (1946) list 15 potential hosts for the larval stage, 16 potential hosts for the nymphal stage, and 29

potential hosts for adults. The species may also be considered unspecialized with respect to habitat since it is found in grassy areas along roadsides, at beaches, and in campgrounds, meadows and fields (Smith et al., 1946). The geographic distribution of D. variabilis ranges from Nova Scotia in the north to the Gulf of Mexico in the south and from the eastern seaboard to the Northcentral and Southwestern states. The species is also found in pockets in Oregon, California, and Hawaii (Sonenshine, 1979). This wide range of geographic distribution suggests a species without strict climactic specialization and further supports the premise that D. variabilis is slowly evolving and not highly specialized, at least in comparison with other tick species.

GC Content

The GC content for D. variabilis duplex DNA is higher than that for most eukaryotes and considerably higher than the comparable value for mammals (49% vs. ca. 40%). In comparison to other arthropods, D. variabilis again has a higher GC content (49% vs. ca. 42%; Sueoka, 1961; Marmur & Doty, 1962). The DNA of D. variabilis should be extremely stable because of this high GC content since the three hydrogen bonds of each GC pair impart a greater structural stability to the duplex than do the two hydrogen bonds between adenine and thymine. Knowledge of

the GC content for duplex DNA's is valuable to the researcher who must interpret molecularly-oriented analyses in nucleic acid biochemistry.

Chromosomal Analysis

Oliver (1972) proposed a completely acrocentric genome for D. variabilis from analyses using uniform staining procedures on reproductive tissue squashes. Prior to the current study, only the sex chromosomes were readily identifiable, principally because of their size. The recognition of a submedian centromere on chromosome six provides the first marker for an autosome in D. variabilis.

Further work on tick genetics could lead to an understanding of the precise mechanisms for viral persistence in ticks and thus an explanation of their transovarial and transstadial transmission. The techniques used in this study may be useful in the basic characterization of the genetic material of other tick species.

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