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Quantitation of nuclear DNA in *Ascaris lumbricoides*: DNA constancy and chromatin diminution

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SUMMARY

The DNA contents of nuclei during gametogenesis and embryogenesis in *Ascaris lumbricoides* were measured by Feulgen-microspectrophotometry. The variability in the mean value for the haploid amount of DNA in sperm from different males processed at different times was not significant when sperm cell samples were taken from the same region of the seminal vesicle. As the sperm mature, the extent of uptake of Feulgen dye decreases nonsystematically. A similar phenomenon occurs during embryogenesis, and as noted by others, primary oocytes in the terminal portion of the oviduct become Feulgen-negative. Feulgen-positive primary oocytes maintain a 4C DNA value without significant variation. Notwithstanding the differences in Feulgen-DNA values in certain types of nuclei, our evidence supports the view that in *Ascaris lumbricoides* the amount of intraspecific DNA has a constant value between individual organisms and from one generation to the next. About 34% of the DNA of the zygote is lost through chromatin diminution at the third embryonic cleavage. This quantity represents 0·23 pg DNA per haploid equivalent.

1. INTRODUCTION

Since its discovery by Boveri (1887), chromatin diminution during the early cleavage stages of *Parascaris equorum* (*Ascaris megalocephala*) has been a provocative example of unusual chromosome behaviour (Wilson, 1928; Davidson, 1968). Recently, it has been shown that the eliminated chromatin from *Ascaris lumbricoides* does not predominantly comprise ribosomal DNA and that the lost DNA contains nucleotide sequences that are not represented in the DNA retained by the somatic cell line (Tobler, Smith & Ursprung, 1972; Tobler, Zulauf & Kuhn, 1974). Furthermore, by isotope dilution, the relative amount of discarded DNA was estimated to be about 27% of the germ-line chromatin (Tobler *et al.* 1972). By contrast, Moritz (1970*a*), using microspectrophotometry, found that 80% of the DNA is eliminated during the development of *Ascaris megalocephala*. This discordance may reflect inherent differences between ascarid species or, as noted by Tobler *et al.* (1972), the isotope dilution method which does not account for somatic polyploidy may, thereby, underestimate the amount of lost DNA.

In addition, a number of Moritz's (1970*b*) other observations are of intrinsic interest. For example, he found that eggs produced by a single female have

significantly different nuclear DNA contents, that after the first cleavage division there is variation in the amount of DNA between homologous chromosomes, that the sperm produced by a single male contain significantly diverse DNA contents, and that the amount of DNA in sperm from different males is significantly heterogeneous. These data suggest that not only does this organism produce large numbers of aneuploid sex cells, but that the so-called rule of DNA constancy may not be stringently maintained in this species. The question arises whether these phenomena are peculiar to *A. megalocephala*. We decided therefore to examine with Feulgen microspectrophotometry the nuclear DNA contents of spermatids, oocytes, and cleaveage stages of *Ascaris lumbricoides*. Moreover, the extent of chromatin diminution was quantified.

2. MATERIALS AND METHODS

Fresh *Ascaris lumbricoides* var. *suum* was obtained from a local slaughterhouse. In the laboratory, adults were separated, grouped into sexes and thoroughly washed in warm deionized water. Individual specimens were submerged in 0·14 M-NaCl, pinned to a dissecting tray and slit longitudinally. The complete reproductive tract was carefully removed, unravelled and measured. Subsequently, each tract was cut into successive 5 cm segments. The relative location of each segment as a function of the total length of the gonad was recorded.

After selecting a representative sample of gonadal pieces from individual worms, the gametocytes were extruded from each segment into a drop of distilled water on separate slides. The cells were dried at 42 °C overnight and post-fixed in absolute ethanol:glacial acetic acid (3:1, v/v) at 5 °C. The remaining intact gonadal segments from any individual worm were fixed whole and retained for embedding.

To obtain various stages of embryogenesis, *Ascaris* eggs were collected from excised uteri of mature females and stored in 0·1 N-H_2SO_4 at 5 °C (Fairbairn, 1955). Embryonic development was induced by shaking the egg suspensions at 30 °C. Samples were withdrawn daily for 10 days, washed free of acid and fixed in absolute ethanol:glacial acetic acid (3:1, v/v) at 5 °C.

Intact segments of the gonads and embryonic stages were embedded in 93·4% purified glycol methacrylate (Sin & Pasternak, 1970). Sections were cut to about 5 μm. Two- and four-celled embryos were sectioned at 8 μm and eight-celled embryos at 6 μm.

Both the whole mount and sectioned material were hydrolysed for 30–35 min in 5 N-HCl at 25 °C (Fand, 1970). The conditions for optimal hydrolysis were regularly checked. Routinely, all the material comprising one complete experiment was processed concomitantly. Chicken red blood cells were used as a standard in all experiments. After hydrolysis, the material was rinsed once in distilled water, immersed in Schiff's reagent for 2 h in the dark at room temperature, decolorized in freshly prepared sulfite bleach, washed with distilled water, air dried and mounted in immersion oil (η_D = 1·568; R. P. Cargille Laboratories, Inc.).

Cytophotometric measurements were made with a modified Leitz MPV microscope photometer (Pasternak & Haight, 1975). The amount of chromophore was

usually determined with the two-wavelength method (Patau, 1952). Spectral absorption curves were plotted for each batch of stained material. The criterion of $E_a/E_b = 0{\cdot}51 - 0{\cdot}53$ (where E_a and E_b refer to extinction values for the Feulgen stain at the lower and higher wavelengths respectively) was often satisfied with 505 and 565 nm. Occasionally, the wavelengths 496 and 555 nm were used. The 'slope zero test' was performed routinely (Garcia & Iorio, 1966). Nuclear areas were determined photometrically (Garcia & Iorio, 1966). In some instances, the 'plug' method was employed (Swift, 1950).

The specificity of the Feulgen stain was tested by incubating preparations with DNase (0·3 mg/ml in aqueous 0·004 M-$MgSO_4$, pH 7·0 for 12 h at 37 °C) prior to hydrolysis. Such a treatment abolished Feulgen positivity in all of the cell types examined.

3. RESULTS

Nuclear DNA contents during spermatogenesis

The male ascarid reproductive tract is an extensively coiled, single tube *in situ* which, when unravelled, extends about 140 cm. Starting from the germinal tip the testis occupies about 100 cm of the gonad or about 72% of the entire length. The next 20% of the gonad consists of the sperm duct which can be demarcated histologically. The terminal 8% of the gonad includes a broad seminal vesicle which empties into the cloaca.

In a number of experiments nuclear DNA contents and areas of gametocytes from discrete portions of the male gonad were ascertained. In Table 1 a representative set of measurements for one adult male gonad is presented. There is an abrupt change in both DNA readings and nuclear areas within the reproductive tract at a point about 80% from the germinal tip. Although meiotic stages could not be unequivocally identified in this portion of the gonad, the reduction of Feulgen dye suggests that complete chromosome reduction occurs in this vicinity of the tract. The mean Feulgen dye content of spermatocytes occupying the region from 8 to 63% of the gonad was 5·88 arbitrary units (AU) and the mean value for presumptive sperm (81–85% of the gonad) was 1·52 AU. These relative readings are not significantly different from a 4 : 1 ratio (χ^2 test, d.f. = 1, $P > 0{\cdot}70$).

As the sperm progress through the gonad, the relative Feulgen dye content diminishes (rows 8–11, Table 1). The DNA values of maturing sperm that are found within terminal sections of the gonad (i.e. from 87 to 100% of the gonadal length) show significant variability (F test; $F = 9{\cdot}772$; d.f. = 3, 175; $P < 0{\cdot}001$). Application of the Sheffé test for multiple comparisons further revealed that the sample of sperm with a mean of 1·08 AU of DNA is significantly different from each of the three sperm samples with mean DNA values that lie between 0·81 and 0·90 AU. The mean DNA values of the latter sperm samples were not found to be different. The relative decrease in the amount of measurable chromophore bound to the DNA of maturing sperm was variable and ranged from 12 to 47% in six experiments. We examined whether the removal of acid-soluble material would alter the binding of Feulgen to the DNA of maturing sperm. Samples of sperm from the terminal portion

of the gonad (95–100% region) were isolated. Half the sample was treated with 0·1 N-HCl for 3 h at 30 °C prior to hydrolysis in 1 N trichloroacetic acid at 60 °C for 14 min and stained with Feulgen (Noeske, 1971). Untreated control samples were processed concomitantly. Extracted sperm yielded DNA values significantly lower than control sperm (Student's t test; $t = 2{\cdot}818$; d.f. $= 28$; $P < 0{\cdot}01$) whereas in hen red blood cells the uptake of dye increased after mild acid hydrolysis (Student's t test; $t = 6{\cdot}41$; d.f. $= 28$; $P < 0{\cdot}01$).

Table 1. *Nuclear DNA contents of male gametocytes of* Ascaris lumbricoides

Gonadal regions (%)*	n	DNA (AU ± S.D.)	Area (μm^2 ± S.D.)
0–3	43	3·38 ± 0·28	20·40 ± 6·55
8–11	43	5·65 ± 0·69	60·75 ± 30·21
22–26	44	5·86 ± 0·60	77·41 ± 18·52
37–41	44	6·32 ± 0·75	76·92 ± 18·91
48–52	45	5·85 ± 0·11	79·86 ± 21·34
59–63	24	5·71 ± 0·31	82·31 ± 18·72
81–85	45	1·52 ± 0·40	3·21 ± 0·84
87–89	45	1·08 ± 0·21	3·13 ± 0·63
90–93	45	0·89 ± 0·25	3·55 ± 0·69
95–97	44	0·90 ± 0·26	3·38 ± 0·48
98–100	45	0·81 ± 0·16	3·24 ± 0·59

* Location of gonadal segments expressed as percent of the total length of the gonad measured from the germinal tip.

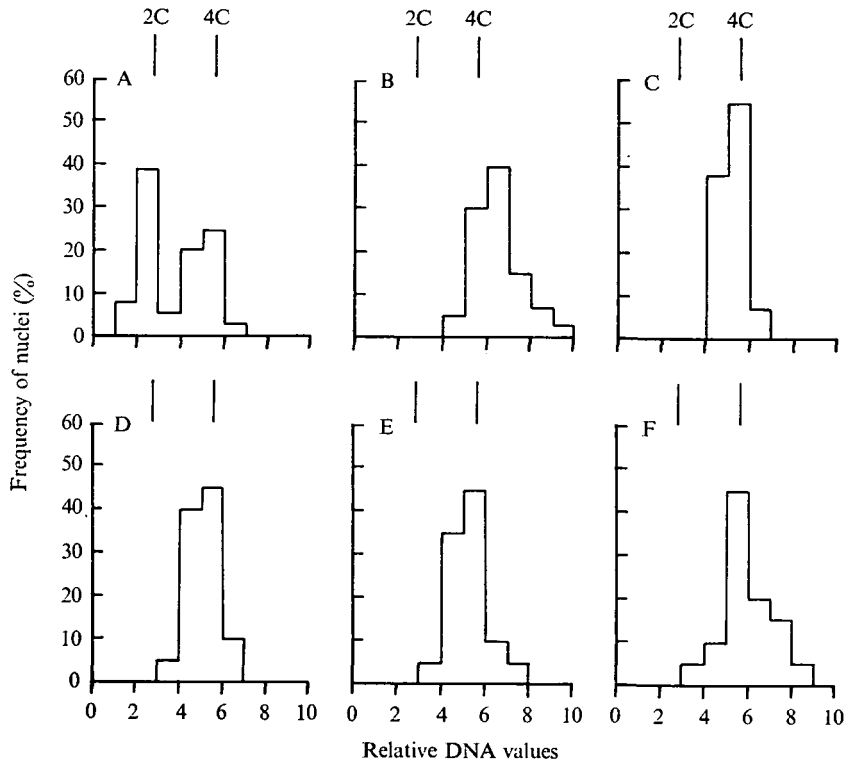
Table 2. *Analyses of DNA content of sperm from different* Ascaris lumbricoides *males: Analysis of variance and Bartlett's test for homogeneity of variance*

	Analysis of variance					
	Source of variation	d.f.	Mean squares	F value	P	Bartlett's test
Example 1	Between males	2	0·3013	3·02	> 0·05	$0{\cdot}95\chi^2_2 = 6{\cdot}0$; $P > 0{\cdot}5$
	Within males	50	0·0999			
Example 2	Between males	4	0·0413	2·24	> 0·05	$0{\cdot}95\chi^2_4 = 1{\cdot}58$; $P > 0{\cdot}90$
	Within males	70	0·0183			
Example 3	Between males	2	0·01999	1·31	> 0·20	$0{\cdot}95\chi^2_2 = 0{\cdot}621$; $P > 0{\cdot}70$
	Within males	52	0·01529			
Example 4	Between males	5	0·02588	0·61	> 0·20	$0{\cdot}95\chi^2_5 = 4{\cdot}76$; $P > 0{\cdot}30$
	Within males	32	0·0425			

The variability of ascarid sperm DNA content from randomly selected mature males was tested on different occasions over a two year period. In none of the experiments did either the means differ significantly or the variances reveal any underlying heterogeneity (Table 2). In each run, care was taken to use mature males of the same overall length and to isolate sperm from the same relative location in the seminal vesicle of the different males.

Nuclear DNA contents during oogenesis

The female reproductive tract of *Ascaris lumbricoides* is opisthodelphic. In mature females, the gonad consists of a pair of extensively coiled tubes. When extended, each branch of the gonad measures about 210 cm. The ovaries occupy the initial 5% of the tract. The oviducts, definable histologically, extend from 6 to 85% of the length of the gonad and the broadened uteri comprise the terminal 15% of the tract.



Text-fig. 1. Frequency distribution of Feulgen-DNA values of female germ cells from various regions of the reproductive tract of *Ascaris lumbricoides*. The 2C value is 2·81 arbitrary units. In each panel the vertical lines denote 2C and 4C values, respectively.

Panel	Region	*n*	Mean DNA (AU)	Nuclear area (μm^2)
A	0–3%	39	3·79 ± 1·38	33·2 ± 6·5
B	15–18%	23	5·98 ± 0·87	39·2 ± 17·4
C	38–40%	24	5·20 ± 0·53	41·1 ± 7·3
D	50–53%	24	5·09 ± 0·67	57·7 ± 12·2
E	61–64%	25	5·20 ± 0·72	68·0 ± 12·7
F	71–74%	25	5·96 ± 1·17	84·6 ± 17·4

Feulgen DNA contents of nuclei within various regions of the female reproductive system were determined (Text-fig. 1). Nuclei at the germinal tip (0–3% region) have a bimodal distribution with 48% of the nuclei having a DNA content that is equivalent to 2C and 47% of the nuclei approximate a 4C value. Primary oocytes with a

4C DNA equivalent occupy the oviduct. At a point about 75% of the length of the gonad from the germinal tip, the oocytes become Feulgen-negative and therefore cannot be measured. There is no evidence that, as the primary oocytes pass along the oviduct, (1) significant changes occur in the nuclear DNA content of the oocytes or (2) the variability in the amount of oocyte DNA increases (Table 3). Although it was not studied in detail, the Feulgen DNA contents of oocytes from different mature females that were processed simultaneously were found not to be significantly different (F test; $F = 2{\cdot}08$; d.f. = 3, 153; $P > 0{\cdot}10$).

Table 3. *Analysis of variance of DNA contents of primary oocytes within regions of the oviducts of mature females of* Ascaris lumbricoides

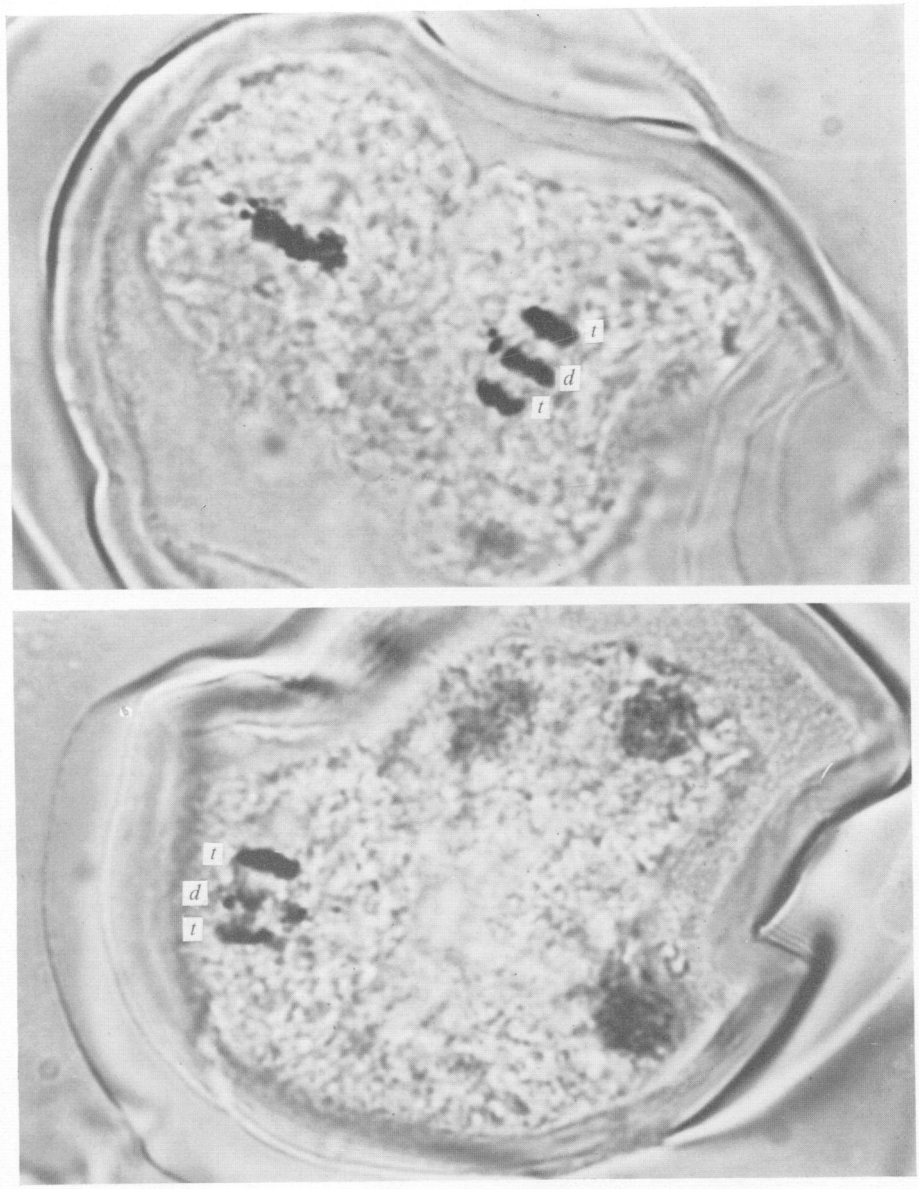
	Source of variation	d.f.	Mean squares	F value	P
Example 1	Between regions	3	0·433	1·66	> 0·10
	Within oviduct region	151	0·261		
Example 2	Between regions	4	5·07	2·26	> 0·05
	Within oviduct region	167	2·24		
Example 3	Between regions	3	0·306	0·35	> 0·20
	Within oviduct regions	56	0·887		
Example 4	Between regions	5	0·305	0·58	> 0·20
	Within oviduct regions	84	0·525		
Example 5	Between regions	2	0·120	0·29	> 0·20
	Within oviduct region	70	0·411		

Table 4. *Nuclear DNA contents during embryogenesis of* Ascaris lumbricoides

Stage	n	DNA (AU ± S.D.)	Area (μm^2 ± S.D.)
2 blastomeres	15	2·89 ± 0·89	60·80 ± 0·23
4 blastomeres	15	2·56 ± 1·38	44·23 ± 0·11
8 blastomeres	15	1·42 ± 0·50	32·16 ± 0·12
~30 blastomeres	15	0·65 ± 0·13	22·19 ± 0·10
Gastrula	15	0·71 ± 0·17	10·11 ± 0·05
L1 juvenile	15	0·19 ± 0·05	4·75 ± 0·43

Nuclear DNA contents during embryogenesis

A Feulgen-positive nuclear reaction is evident at the 2-celled stage of embryogenesis and thereafter. As the embryo develops the nuclear areas decrease about 10-fold and the amount of nuclear-bound Feulgen dye diminishes considerably (Table 4). This cytophotometric trend has been observed in four experiments when measurements were obtained by the two-wavelength method and in three experiments with the plug method. The apparent hypodiploidy of the somatic nuclei of L1 juvenile worms averages about 25% of the expected 2C value of a postdiminution somatic cell. Extraction with mild acid (0·1 N-HCl) prior to hydrolysis with trichloroacetic acid and staining with Feulgen did not alter the binding of the chromophore to nuclei of any of the embryonic stages.



Examples of chromatin diminution during the third cleavage mitosis in *Ascaris lumbricoides*. Feulgen stain. *t*, telophase; *d*, diminuted chromatin. Magnification about × 3200.

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Quantitation of chromatin diminution

In *Ascaris lumbricoides* chromatin diminution begins at the third embryonic cleavage. During this division, three discrete bands of Feulgen-positive material are evident in those sections with a fortuitous plane of cut (Plate 1). In seven such cases, readings were taken from the eliminated chromatin and both of the third cleavage telophases (Table 5). Primary spermatocytes and immature sperm that had been processed concomitantly served as standards. From these data, it is estimated that the somatic cell line after the third cleavage has about 34% less chromatin than is retained by the germ line cells. Using hen red blood cells as a standard (Rasch, Barr & Rasch, 1971), the absolute amount of DNA per *Ascaris* sperm was found to be $0{\cdot}68 \pm 0{\cdot}15$ pg (mean $\pm$ S.D. of 4 determinations). Therefore a total of about 0·92 pg DNA is lost during the third embryonic mitosis in this organism.

Table 5. *Chromatin diminution in* Ascaris lumbricoides

Stage	*n*	DNA (AU $\pm$ S.D.)
Telophase groups, 3rd cleavage	14	$1{\cdot}29 \pm 0{\cdot}21$
Eliminated chromatin, 3rd cleavage	7	$1{\cdot}35 \pm 0{\cdot}40$
Primary spermatocytes	15	$4{\cdot}06 \pm 0{\cdot}36$
Immature sperm	15	$1{\cdot}04 \pm 0{\cdot}14$

4. DISCUSSION

This investigation was undertaken to determine: (1) the extent of variability in the modal DNA contents of various nuclear types of *Ascaris lumbricoides* and (2) the quantity of DNA that is lost during the third cleavage of embryogenesis in this species. Sperm DNA values in samples from males processed at different times did not show significant heterogeneity. This observation contrasts with Moritz's findings for *Ascaris megalocephala* (1970*b*). In *Ascaris lumbricoides*, as the sperm matures the extent of uptake of the Feulgen chromophore diminishes due, presumably, to some form of DNA compaction (e.g. Gledhill, Gledhill, Rigler & Ringertz, 1966). Thus, if all of the developing sperm in an ascarid male were pooled and treated experimentally as a homogeneous population it is conceivable that an apparent heterogeneity of DNA values would result. We do not know if such an explanation accounts for the variability of sperm DNA contents in *A. megalocephala*. In addition, we found no evidence for the large-scale production of aneuploid ova nor did the DNA contents of oocytes from different females that were processed concomitantly vary significantly. Therefore, these data are consistent with the view that intraspecific DNA constancy is maintained in *Ascaris lumbricoides*.

Periodic changes in the pattern of Feulgen staining of various nuclear types during both oogenesis and embryogenesis of different nematode species has been noted previously (Pasteels, 1948; Lin, 1954; Monné, 1963). Moreover, in other organisms, the stoichiometry of the Feulgen-DNA reaction varies in diverse cell types and at various stages of cellular differentiation (Noeske, 1971; Mayall, 1969; Rasch & Rasch, 1973, and references therein). Noeske (1971) found that an

equivalence of Feulgen cytophotometric values in different bone marrow cell types could be restored by extracting acid-soluble material prior to staining. In our case, when this treatment was applied to mature sperm, it caused a further decrease in Feulgen DNA values, whereas it was without effect on the DNA values of the late embryonic stages. Furthermore, in *Ascaris* the reduction in Feulgen dye readings correlates with the disappearance of both arginine- and lysine-rich proteins from the nuclei (our unpublished results). It is difficult to account for the anomolous behaviour of the Feulgen technique. On the one hand, it can be argued that the changes in DNA values during spermiogenesis are probably not due to a biologic loss of DNA *per se* since blastomeres of developing embryos, prior to chromatin diminution, have the characteristic 2–4C ploidy levels. Indeed, for such a proposition to be tenable the organism would have to possess a special compensatory mechanism that re-establishes the intraspecific level of DNA during each generation. Otherwise, one is faced with a *reductio ad absurdum*, namely the DNA content of *Ascaris* is progressively decreased during successive generations. On the other hand, some variability in the absolute amount of DNA per nucleus is feasible during the development of somatic cell lines; especially in an organism that undergoes chromatin diminution. We have no direct evidence to support this contention since differences among Feulgen dye values can be explained in a number of ways (Rasch & Rasch, 1973). Others have found that somatic cell types of *Ascaris* may be either polyploid or Feulgen-negative (Swartz, Henry & Floyd, 1967). It was not determined whether the distributions of DNA contents of the polyploid cells conform to a geometric series representing multiples that are equivalent to a post-diminution DNA value. Our results only indicate that somatic polyploidization in *Ascaris* occurs during postembryonic development. Moreover, Feulgen-negativity does not necessarily denote the absence of DNA (Zajicek, Swartz & Floyd, 1970). In sum, it seems that certain variations of Feulgen dye uptake depend upon the configuration of the nuclear DNA, the presence of nuclear proteins and/or the degree of differential extraction of DNA components rather than changes in the quantity of nuclear DNA *per se*. In this context, interpretation of discrepant intraspecific Feulgen-DNA values must be handled with circumspection.

The DNA equivalent of a haploid spermatid amount in *Ascaris lumbricoides*, determined by chemical analysis (Tobler *et al.* 1972) and approximated from DNA renaturation kinetics (Searcy & MacInnis, 1970) is about 0·63 pg and 0·25 pg respectively. Our estimate of the genome size of *A. lumbricoides*, based on Feulgen microspectrophotometric readings of presumptive sperm compared to hen erythrocyte nuclear DNA content as a reference, is $0{\cdot}68 \pm 0{\cdot}15$ pg. Furthermore, our data show that the difference in DNA contents between pre- and post-diminution blastomeres is 34 %. This decrease in DNA content agrees well with the 27 % value presented by Tobler *et al.* (1972). It is unlikely that differential binding of Feulgen stain influenced our estimate. For example, a summation of the DNA values for both telophases and the chromatin left at the metaphase plate during the third cleavage approximates a 4C value when either spermatocytes or immature sperm are used as standards. In comparison, *A. megalocephala* discards about 80 % of its chromatin

(Moritz, 1970*a*) and in *Cyclops furcifer*, a copepod, the amount eliminated is about 70% of the pre-diminution chromatin (Beermann, 1966). If the eliminated DNA in these organisms represents genetic information that is predominantly concerned with gametogenesis and ancilliary germ-line functions, then one must conclude that a considerable preponderance of the overall genome is devoted to these mechanisms. For example, in *A. lumbricoides* the loss of 0·23 pg per haploid equivalent represents about $2{\cdot}2 \times 10^8$ nucleotide pairs. Since about half of the eliminated DNA in *Ascaris* contains 'unique' sequences (Tobler *et al.* 1972), then chromatin diminution results in the loss of sufficient nucleotide pairs to code for $1{\cdot}1 \times 10^5$ different proteins. Certainly, a case can be made that part of the diminuted DNA includes germ-line specific genetic information; but it is difficult to rationalize the content of all the eliminated DNA in this manner. Accordingly, both the significance and the cause of chromatin diminution remain obscure. In this context, the ciliated protozoan *Stylonychia* which can dispense with 93% of its original micronuclear DNA and continue to thrive after amplification of the remaining DNA portion provides a striking example of the minimal DNA requirement that is compatible with maintaining ongoing somatic cell functions (Prescott, Murti & Bostock, 1973).

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