DNA IN GAMETOGENESIS AND EMBRYOGENY IN TRADESCANTIA*

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Plates 210 and 211

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Within recent years evidence has come from several lines of research that the gene is either composed of, or is at least intimately associated with, desoxyribose nucleic acid (DNA). Experiments in microbial genetics have shown that only the DNA fraction of the bacterial protoplast is capable of transforming other bacteria to the genetic type from which the DNA was originally derived (2). Further, both chemical extraction and photometric studies have supported the view that within a plant or animal the amount of DNA per nucleus occurs in simple multiples of a basic (haploid) amount (see references 30, 34 for review). Such consistent quantitative behavior could account, at least in part, for the remarkable genetic constancy of successive cell generations and has been presented as evidence for close relationship between DNA and the stable characteristics of the gene (see reference 30 for review).

The nucleus has been known for some time to embody several other forms of stability. Studies on uptake of radioactive phosphorus (P^{32}) have shown a striking metabolic stability in the DNA molecule. Autoradiographic studies (see references 11, 33, 16, 8 for review) indicate that although incorporation of P^{32} into DNA is high in tissues where DNA synthesis is occurring, it is very low in some non-dividing tissues. Furthermore, chromatographic separation and quantitation of the purines and pyrimidines of DNA indicate that the proportions of the purine and pyrimidine bases seem to be constant for a species (6, 35). In contrast to the stability of DNA, ribose nucleic acid (RNA) and some protein fractions appear to vary and Ris (22) has suggested that constancy of DNA represents nuclear stability presumably related to the stable aspects of the gene, while the variability of some protein and RNA fractions represents instability and change in the nucleus according to cell function.

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However, it should be noted that there is not yet unanimity of opinion on the question of DNA "constancy." It has been tempting to consider differentiation as a function of differential reduplication of genic material (DNA) at one or more gene loci (12). Differential polyteny of this kind could result in values intermediate between multiples of the basic DNA amount predicted by the "constancy" hypothesis and such intermediate values have been reported in *Tradescantia* (24, 5). In the light of these considerations it is essential to know which chemical components of the nucleus show constant and which show variable quantitative behavior. Before the growing mass of cytochemical data can be interpreted in terms of causal relationships, controversial points must be critically examined. The purpose of the present study is to reinvestigate, by cytophotometric measurement of Feulgen dye (DNA) content, the nuclei of several plant tissues, the DNA content of which is in dispute, and to present data on tissues not yet discussed in the literature.

Material and Methods

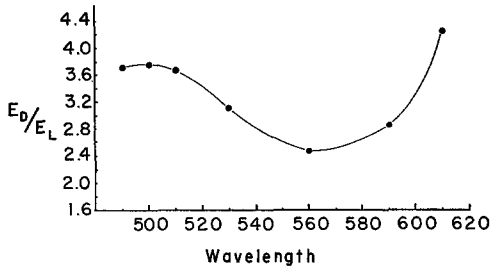
All plant material used in this investigation came from a clone of *Tradescantia paludosa* propagated by cuttings from a single stem. Tissues were fixed in 3:1 absolute ethanol, glacial acetic acid. To aid rapid penetration of the fixative, material was exposed as much as possible by dissection, and was aspirated during fixation. After fixation the material was dehydrated and cleared in an ethanol-benzene series and embedded in paraffin. Sections were cut at thicknesses appropriate to the nuclear size and stained by the Feulgen technique. In order to minimize temperature gradients, slides were preheated in distilled water at 60°C. (9), prior to hydrolysis in 1 N HCl at 60°C. for 8 minutes. Tissues were mounted in harleco synthetic resin (refractive index 1.540). The photometric apparatus used was essentially similar to that described by Pollister and Ris (21) except that light was isolated by a monochromator designed by Patau (data to be published) for use at the microscope.

In dealing with nuclei of widely different stainability, the wave length of light chosen for measurement must represent a compromise. It should not give extinctions so low that lightly stained nuclei are overestimated because of non-specific light loss nor should it be so high that distributional and stray light errors cause serious underestimates of measured amounts of dye in deeply stained nuclei (for a full discussion of these errors see reference 32). Moreover, the photometric apparatus may vary in its efficiency according to the wave length used. The following test was devised to determine the capabilities of a given instrument at various wave lengths. The extinctions of a heavily absorbing nucleus and a lightly absorbing nucleus on the same slide were compared at a number of wave lengths. Assuming that the light and dark nuclei have the same absorption curve, the ratio of extinctions of these nuclei should be fairly constant at all absorbing wave lengths except where it is modified by the errors mentioned above. It is thus possible to assign specific errors to changes in the extinction ratio. Stray light error or distributional error, or both, in the region of peak absorption can considerably reduce the extinction ratio by underestimating the absorption of the dark nucleus. A decreased extinction ratio in the blue and red regions of the spectrum, on the other hand, indicates nonspecific absorption, which overestimates the extinction of the lightly stained nucleus more than the darkly stained nucleus. This latter error which is often due to light scatter, particularly in the shorter wave lengths, can be reduced by mounting in a refractive index oil which matches the refractive index of the tissue. Pairs of extinctions of light and dark nuclei can be plotted in other ways. For example, the test has been modified by Swift and Rasch (32) who

prefer either to plot the extinctions against one another or simply compare error-induced differences in the two absorption curves.

The test was applied to the present instrument using two nuclei, a very darkly stained one from the outer integument, and a very lightly stained one situated between the outer integument and ovary wall. These nuclei were found a few microns apart in a section of ovary fixed 3 days after pollination. The ratio of their extinctions, E dark/E light is plotted against wave length in Text-fig. 1. The curve shows a sharp dip in the mid region indicating that the instrument had considerable stray light error at these wave lengths. Error seems to be minimum between 4900 and 5100 A. Consequently, a wave length of 5000 A was selected for measurement. At this wave length mean extinctions spread between 0.204 for eggs and 0.910 for endosperm.

Amounts of Feulgen dye were expressed as ED^2 in which D is the diameter of the nucleus in arbitrary units, and E the extinction of a small plug (one-third or less the nuclear diameter) through the center of the nucleus. At least two replications per nucleus were made, as suggested by Patau (19, 20), in order to reduce errors resulting from small electrical irregularities throughout the system (photometric noise). Variation from this source can be minimized by rejecting values derived from pairs of transmissions whose difference exceeds an arbitrary



TEXT-FIG. 1. The extinction ratio of a darkly stained nucleus E_D and a lightly stained nucleus E_L plotted against wave length.

amount, which in the present investigation is 0.01. This arbitrary standard insures that that portion of non-biological variation attributable to noise is minimized (for statistical consideration see reference 27).

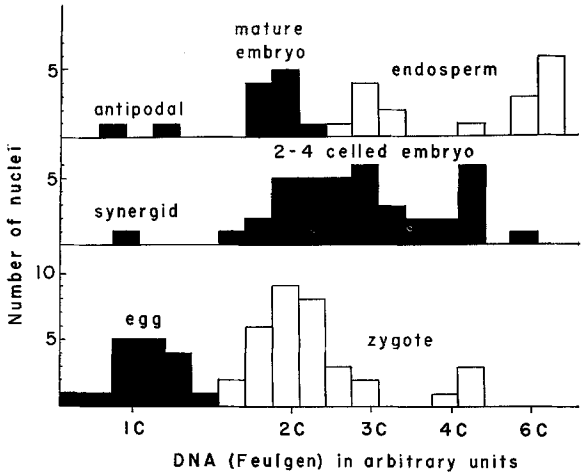
OBSERVATION AND RESULTS

(a) *Embryo Sac and Embryo Stages.*—Microphotometric measurements of material fixed on both the day before and the day of flowering indicate that eggs, synergids, and antipodals contain the lowest or basic (1 C)[1] amount of DNA found in the plant (Table I, Figs. 1 and 2).

On the day following pollination, fertilization has occurred in the case of both egg and polar nuclei. Union of gametes must be rapid for fertilization stages are relatively rare. Measurable microgamete nuclei were sought in pollen tubes grown on 8 per cent sucrose (15), and within squashed or sectioned styles fixed at intervals of several hours following pollination. In all

[1] The terminology of Swift (29) will be used to designate DNA amounts, *i.e.* 1 C represents the unitary amount of DNA, 2 C twice this amount, etc.

instances microgamete nuclei were found unmeasurable because of their extreme elongation. Microgamete nuclei were also observed in the embryo sac and were found to take several forms. They exist as an extremely elongate, coiled, longitudinally divided structure lying alongside the egg (see Figs. 3 *a*, *b*, *c*, *d*), or at other times as a small compact nucleus pressed against the egg (Fig. 4). Gerassimova (10) has reported that the microgamete nucleus of *Crepis* also consists of two longitudinal halves which are distinguishable even when the microgamete forms a ball-shaped body. It is possible that the bipartite nature of the 1 C chromosome such as has been reported for the anaphase chromosomes of some plants is in evidence here (for review see reference 13), the chromosomes taking an end to end arrangement within the narrow



TEXT-FIG. 2. Frequency distribution of relative amounts of DNA in embryo sac nuclei of *Tradescantia*. Scale of abscissa is logarithmic.

pollen tube. Later the zygote nucleus becomes spherical although the microgamete component is still recognizable as a densely stained zone of the zygote nucleus (Fig. 5).

Two days after pollination the egg and microgamete components of the zygote nucleus are no longer visually distinguishable. Most of these zygote nuclei are still in the 2 C DNA class with a few intermediates and an occasional 4 C nucleus appearing (Figs. 6 and 7). Although in the mouse (1) and in the grasshopper (31) DNA synthesis occurs in the pronuclei before actual nuclear union, in *Tradescantia* it is clear that synthesis follows this union. Three days after pollination most of the embryos are in the two and four cell stages (Figs. 8 and 9). Text-fig. 2 shows that DNA values in these stages range between the 2 C and 4 C amounts. The next stage considered was the late embryo which was dissected from shed seed (Fig. 10). All nuclei measured have the 2 C amount of DNA (Text-fig. 2).

The development of the endosperm differs greatly from that of the embryo. The endosperm is derived from the triple fusion of one microgamete and two polar nuclei, each of which is up to four times the size of the egg nucleus. This large nuclear size is possibly an expression of the accumulation of substances which will be required for an immediate surge of mitoses following fertilization. Certainly, fertilization must induce a relatively rapid series of divisions in the endosperm, for at a time when the one celled zygote is still 2 C the

TABLE I

Relative Amounts of DNA in Individual Nuclei in Various Tissues of Tradescantia

Cell type	Mean relative amount of DNA and standard error (or in dividing tissues, modes when present)	DNA class	Number measured
Egg	5.18 ± 0.14	1 C	17
Zygote	9.44, 21.1 (modes)	2 C–4 C	34
2–4 celled embryo	21.1 (mode)	2 C–4 C	38
Mature embryo	9.01 ± 0.19	2 C	10
Endosperm	13.3, 29.8 (modes)	3 C–6 C	17
Antipodal	5.12, 4.19	1 C	2
Synergid	4.81	1 C	1
Pollen mother cell	18.21 ± 0.43	4 C	11
Early microspore	4.79 ± 0.09	1 C	10
Late microspore	9.41 ± 0.18	2 C	10
Early generative*	4.73 (mode)	1 C	10
Mid generative*	9.44 (mode)	2 C	10
Early vegetative*	5.14 ± 0.10	1 C	10
Late vegetative*	5.64 ± 0.14		10
Ovary wall	8.95 ± 0.16	2 C	10
	17.93 ± 0.46	4 C	12
Elongation and differentiated zone of root	9.90 ± 0.32	2 C	11
	19.5, 18.5	4 C	2
Root meristem	—	2 C–4 C	36
Leaf	9.07 ± 0.17	2 C	10

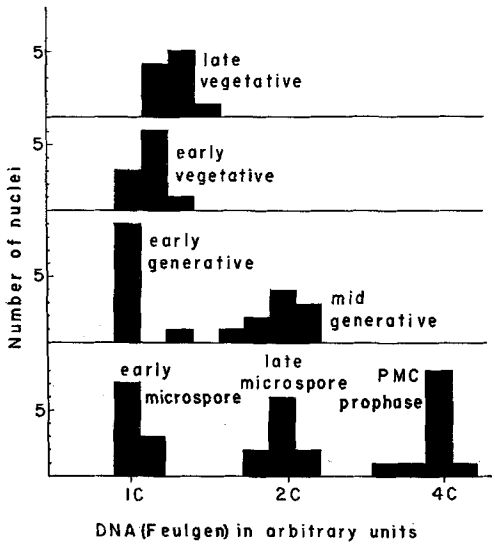
* See Text-fig. 4 and Table II for remeasurements of these nuclei at 6000 A after remounting in a matching refractive index oil.

endosperm is approximately 14-nucleate, and during the two celled embryo stage there are up to 100 endosperm nuclei.

As one might expect from the triple genomic constitution of the endosperm, DNA values scatter between the 3 C and 6 C amount in material fixed up to and including 7 days after pollination (Text-fig. 2). The higher multiples of the 3 C amount found in corn (29) and in *Tradescantia* (Rasch, Swift, and Nagaraj, data to be published) were not found. These stages probably occur in *Tradescantia* in older ovules where DNA synthesis without intervening mitoses has begun.

(b) *Microsporogenesis.*—In general microsporogenesis was found to follow

the pattern described by Swift (29), Pasteels and Lison (18), and Moses and Taylor (16). The earliest stages analyzed in the present study are late prophases of pollen mother cells (PMC), which contain the 4 C amount of DNA (Table I and Text-fig. 3). Meiotic division of these nuclei gives rise to quartets of early microspores each containing the 1 C amount of DNA. The microspore nucleus then moves from the center of the cell to the end and finally back to the center again, the movement being accompanied by a great increase in nuclear size. In this final central position DNA synthesis occurs, bringing the late microspore nucleus to the 2 C level. The first gametophytic mitosis then takes place to give rise to two 1 C nuclei, a small oblately sphe-

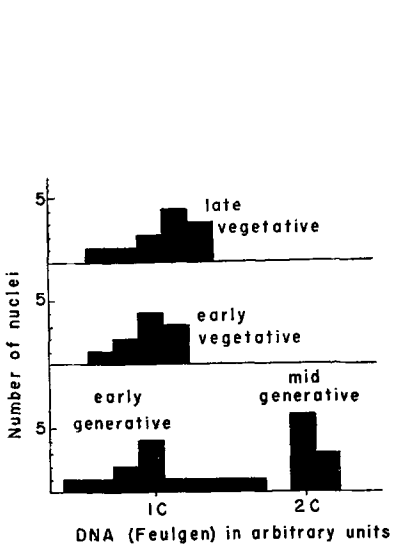


TEXT-FIG. 3. Frequency distribution of relative amounts of DNA in several stages of *Tradescantia* microsporogenesis. Scale of abscissa is logarithmic.

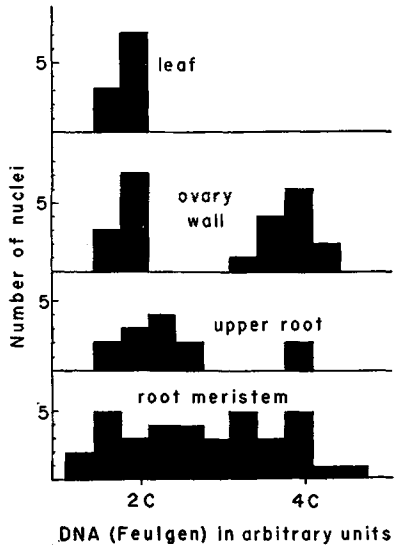
roidal one which is the generative, and a large spherical one which is the vegetative.

As development proceeds, the generative nucleus increases to the 2 C value, while the mean DNA value for vegetative nuclei appears to be intermediate between the 1 C and 2 C amount. The increasing light scatter found in the exine and in the cytoplasm and nucleoli of vegetative cells in maturing pollen grains suggested that the observed intermediate value might be attributable to non-specific light loss. This possibility was tested by removing the harleco synthetic resin, mounting in oil of refractive index 1.572, and remeasuring at a wave length of 6000 A on the microphotometric apparatus of H. Swift (32). Matching the refractive index of the tissue with the proper refractive index oil and using a longer wave length for measurement tend to mini-

mize error due to light scatter. It might be expected that vegetative nuclei, whose extinctions were approximately half those of the generative, would be overestimated more than the generative, for a given amount of non-specific light loss will represent a greater percentage of the total absorption in lightly stained (low extinction) nuclei than in darkly stained (high extinction) nuclei. It may be seen from the remeasurements shown in Text-fig. 4 and Table II that these expectations are realized: while the DNA values for early and late generative nuclei are unchanged, both early and late vegetative values



TEXT–FIG. 4.



TEXT–FIG. 5.

TEXT-FIG. 4. Frequency distribution of remeasurements of DNA from the same slide measured in Text-fig. 3, after remounting in a matching refractive index oil. Scale of abscissa is logarithmic.

TEXT-FIG. 5. Frequency distribution of relative amounts of DNA in several somatic tissues of *Tradescantia*. Scale of abscissa is logarithmic.

are lower. The early vegetative nuclei show approximately the same amount of DNA as the early generative, and half the amount of the mid generative. Although the late vegetative nuclei show a 10 per cent increase over the early vegetative, they fall within the range of the 1 C class. Apparently the original measurements of vegetative nuclei were overestimated by non-specific light loss due to scatter while the generative values were relatively unaffected.

It is noteworthy that Moses and Taylor (16) have reported intermediate DNA values (1.8 times the 1 C amount) for vegetative nuclei of *T. paludosa*. Since Sax (23) has shown that the vegetative nucleus of *Tradescantia* is capable of mitosis when subjected to a temperature of 35° C., a common sum-

mer greenhouse temperature, it is conceivable that vegetative nuclei showing intermediate values are in the process of DNA synthesis for such a mitosis.

The great elongation of the late generative nuclei within both the pollen grain and the pollen tube precludes "plug" measurements at these stages. However, it can be inferred that the generative nucleus maintains the 2 C amount of DNA, and divides within the pollen tube to give two 1 C microgametes, for the egg shows an increase after fertilization equal to the 1 C amount of DNA. Moreover, the 3 C endosperm probably originates from the union of two 1 C polar nuclei and a 1 C microgamete.

(c) *Other Somatic Nuclei.*—Several somatic tissues, both dividing and nondividing, were measured and their distribution graphed in Text-fig. 5. Only 2 C nuclei were found in the leaf, while the elongation and differentiated zone of the root as well as the ovary wall, contained two DNA classes, 2 C and 4 C. Although measurements of root meristems often show two frequency

TABLE II

Remeasurements of DNA Amounts in Individual Nuclei of Pollen Grains after Mounting in Refractive Index Oil 1.572

Cell type	Relative amount of DNA	Standard error	DNA class	Number measured
Early vegetative	4.62 (mean)	±0.18	1 C	10
Late vegetative	5.13 (mean)	±0.19	1 C	11
Early generative	4.79 (mode)	.....	1 C	11
Mid generative	9.56 (mode)	.....	2 C	10

maxima, one at the 2 C and another at the 4 C level (29), occasionally the curve shows no significant peaks, as in the present sample. A similar curve has been obtained (26) with onion root meristem. The significance of these differences in the relative numbers of intermediate, 2 C and 4 C nuclei is not known. Such changes might result from a diurnal cycle of mitotic activity.

DISCUSSION

The data presented above are in agreement with the hypothesis that DNA amounts parallel the number of chromosome (or chromatid) sets and are thus "constant." This concept has found support in both biochemical and microphotometric investigations (for review see references 30 and 34). The nuclei studied in the present investigation, except those of dividing tissues, contain approximate multiples of a basic amount of DNA. In tissues with cell divisions present, DNA synthesis results in some values falling between the 1 C and 2 C level in gametophytic tissue and between the 2 C and 4 C level in sporophytic tissue.

This study gives additional support to the general picture of DNA patterns

in gametogenesis and development derived from microphotometric work on both plants and animals. Meiosis is characterized by two divisions of a 4 C nucleus to yield four 1 C nuclei, which either develop directly into gametes as in animals, or undergo several mitoses before gametes are produced, a condition found in plants. Union of 1 C gametes then gives rise to the 2 C zygote, and development proceeds in the pattern typical of dividing tissue: 2 C nuclei build up to the 4 C level of DNA during interphase, halving taking place at nuclear division to give two telophase groups, each containing the 2 C amount of DNA. This quantitative stability is remarkably resistant to many treatments (30), although two recent microphotometric studies have reported an experimental modification of DNA amounts. Setterfield and Duncan (25) have described a 10 per cent reduction in DNA content in root tip nuclei of *Vicia faba* after treatment with a mixture of diaminopurine and adenine, and LaCour *et al.* (14) have reported up to 25 per cent reductions in DNA amounts in several liliaceous plants subjected to cold treatment. Moreover, Schrader and Leuchtenberger (24) and Swift and Kleinfeld (31) have cautioned that some intermediates may reflect certain stable physiological states rather than a premitotic or an endomitotic synthesis. However, available autoradiographic studies in conjunction with microphotometric measurements of DNA (33, 16) indicate that those periods during microsporogenesis in lily and *Tradescantia* in which there are intermediate DNA amounts coincide with the period of incorporation of P^{32} into DNA.

Despite the considerable evidence in favor of the "constancy" concept, it has however, not been universally accepted as much microphotometric data on *Tradescantia* (24, 5) do not support it. However, these data were collected on heavily stained nuclei and measured near the peak of the Feulgen absorption curve. As has been pointed out (30, 32, 16) the reliability of measurements made under these conditions is often questionable because of the likelihood of increased stray light and distributional errors. Also in disagreement with the present findings are results obtained by Ogur *et al.* (17), who used chemical extraction procedures on anthers of *Lilium longiflorum*. They report that the binucleate pollen grain at anthesis contains 7 times, the late microspore 4.7 times, and the pollen mother cells 4.8 times the amount of DNA found in the earliest microspore. Each nucleus, the vegetative and the generative, was considered to contain equal quantities of DNA or 3.5 times the 1 C amount. However, Swift (30) has presented microphotometric data on the same strain of lily used by Ogur *et al.* indicating that the late generative and vegetative nuclei are 2 C and 1 C respectively.

Bryan (5) working with *T. paludosa* obtained microphotometric results that tend to agree with the interpretation of Ogur *et al.* (17). Having found the diploid amount of DNA (Feulgen) in early generative nuclei and the triploid amount in the late vegetative nuclei, he discusses the possibility that both

vegetative and generative build up to the tetraploid amount of DNA. At the second gametophytic division, the generative nucleus would then give rise to two microgamete nuclei, each containing the diploid amount of DNA. The union of such a microgamete with a 2 C egg would result in a 4 C zygote. That is to say, DNA synthesis in preparation for the first division of the zygote would occur in the gametes before syngamy. The data here presented do not agree with this pattern. Not only are late microspore and late generative nuclei 2 C rather than 4 C, and eggs 1 C instead of 2 C, but the early zygote is 2 C indicating it arose from the combination of 1 C gametes.

It should be kept in mind that all photometric studies of the type described here depend on quantitative proportionality between Feulgen dye and DNA present. Swift (28, 30) discusses the evidence bearing on this problem and concludes that when properly handled, the Feulgen stain can serve as a specific, quantitative, cytochemical test for DNA. This, however, does not rule out the possibility that DNA can exist in different qualitative states in different cells or tissues and yet still give a similar Feulgen reaction. Indeed, some recent evidence points to a chemical and metabolic heterogeneity of DNA. Bendich (3) reports two DNA fractions from the rat differing not only in isotope incorporation, but in solubility in saline. Moreover, DNA fractions from a single species have been reported to show differing degrees of binding to histone with variation in salt concentration (7, 4). However, the biochemical data of Chargaff (6) and Wyatt (35) indicate that, at least at the level of purine and pyrimidine bases, the over-all composition of a single species of DNA is constant.

CONCLUSION

In addition to the numerical, morphological, genetic, and metabolic stability generally attributed to the chromosomes, microphotometric studies of DNA content from a variety of tissues in *Tradescantia paludosa* offer evidence of a quantitative cytochemical stability as well. Such stability is appropriate to a gene-bearing organelle and suggests that DNA is closely associated with the gene or that part of it concerned with genetic stability. If there exists a quantitative variability of DNA related to cellular differentiation, such variability is too minute to be detected by present methods of analysis.

SUMMARY

A microphotometric study of various Feulgen-stained gametophytic and sporophytic nuclei of *Tradescantia paludosa* was made to test the hypothesis that DNA is maintained in multiples of a basic unit within all cells of an organism.

A multiple relationship was found in all tissues analyzed. The lowest amount

of DNA was found in gametophytic nuclei, approximately twice and four times this amount in sporophytic nuclei, and approximately three and six times this amount in endosperm nuclei. Intermediate amounts of DNA were found only in tissues presumably undergoing an interphase synthesis of DNA preceding either cell division or endomitosis.

It is concluded that within the limitations of present methods of measurement, DNA amounts show a quantitative behavior which supports the "constancy" hypothesis. Such quantitative stability of DNA gives support to the concept that DNA is associated with the stable elements of the gene.

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EXPLANATION OF PLATES

Several stages in embryo sac and embryo development in *Tradescantia paludosa*. All magnifications are 950, except Fig. 10 which is 200.

A, antipodal nucleus. M, microgamete nucleus.
E, egg nucleus. P, polar nuclei.
EN, endosperm nucleus. S, synergid nucleus.
Z, zygote nucleus.

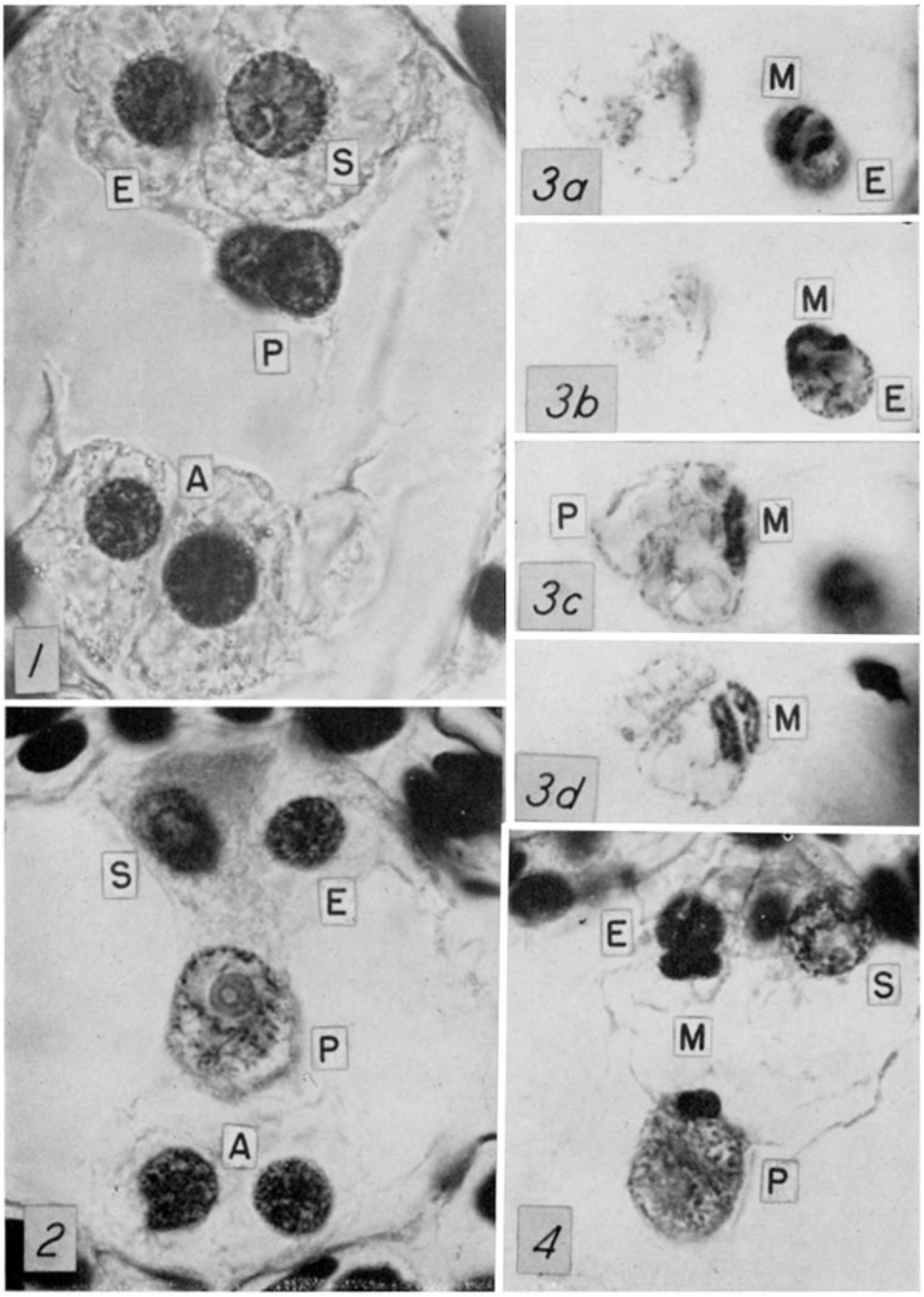
PLATE 210

FIG. 1. Eight nucleate embryo sac showing 6 of the 8 1 C nuclei.

FIG. 2. Later embryo sac fixed on the day of pollination.

FIG. 3 *a*, *b*, *c*, *d*. Double fertilization stage at several focal levels. The 2-partite microgamete is best shown in Fig. 3 *d*.

FIG. 4. Double fertilization stage.



(Woodard: DNA in gametogenesis and embryogeny)

PLATE 211

FIG. 5. Zygote, with recognizable microgamete (see arrow) and egg components. The endosperm nucleus is in prophase of its first mitosis.

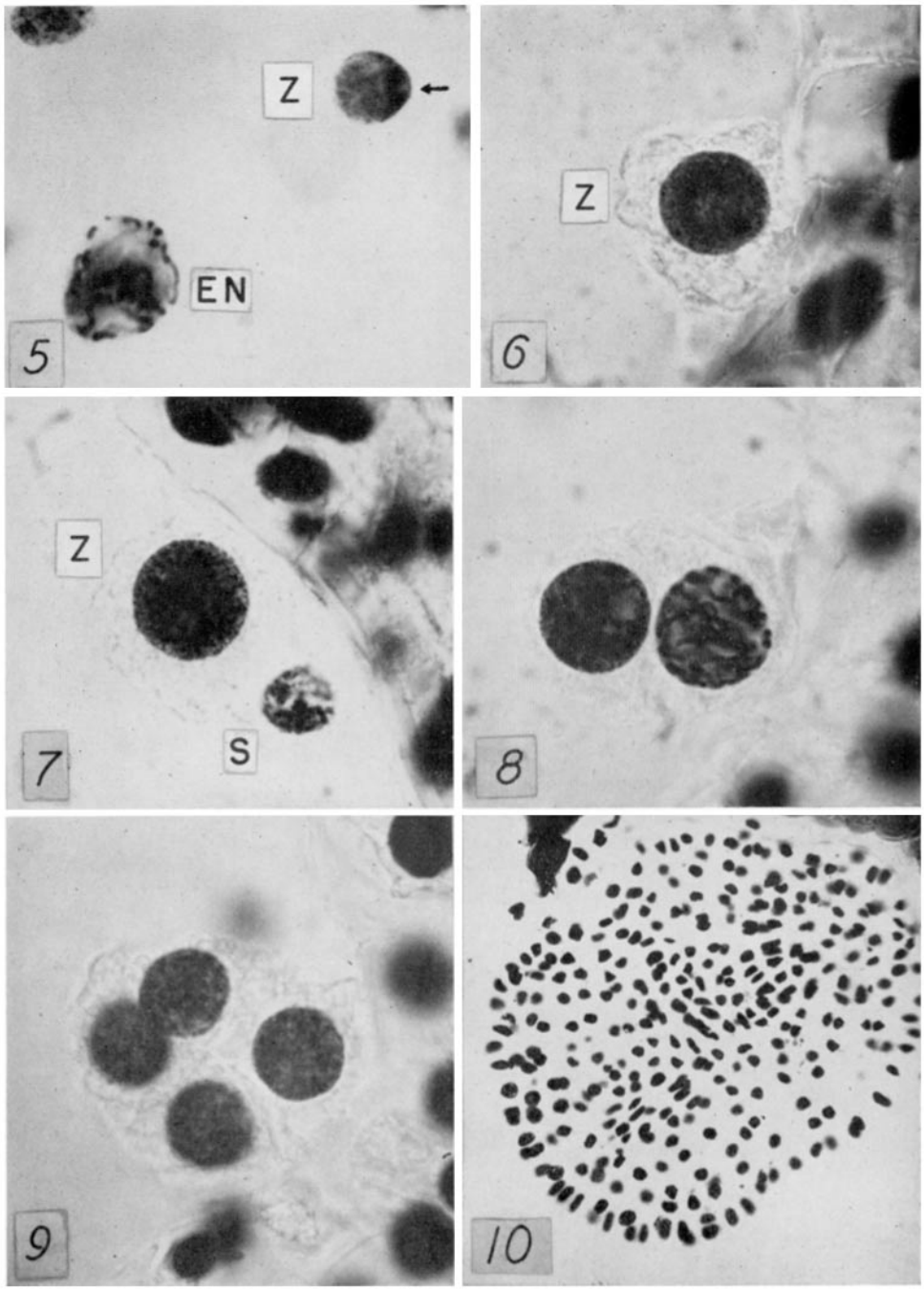
FIG. 6. Zygote nucleus with 2 C amount of DNA.

FIG. 7. Zygote nucleus with 4 C amount of DNA.

FIG. 8. Two celled embryo.

FIG. 9. Four celled embryo.

FIG. 10. Late embryo taken from shed seed.



(Woodard: DNA in gametogenesis and embryogeny)