Basic Proteins of Plant Nuclei during Normal and Pathological Cell Growth*

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PLATES 140 TO 142

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ABSTRACT

Histone proteins were studied by microphotometry of plant tissue sections stained with fast green at pH 8.1. For comparative purposes the Feulgen reaction was used for deoxyribose nuclei acid (DNA); the Sakaguchi reaction for arginine; and the Millon reaction for estimates of total protein.

Analysis of *Tradescantia* tissues indicated that amounts of nuclear histone fell into approximate multiples of the gametic (egg or sperm) quantity except in dividing tissues, where amounts intermediate between multiples were found. In differentiated tissues of lily, corn, onion, and broad bean, histones occurred in constant amounts per nucleus, characteristic of the species, as was found also for DNA. Unlike the condition in several animal species, the basic proteins of sperm nuclei in these higher plants were of the histone type; no evidence of protamine was found.

In a plant neoplasm, crown gall of broad bean, behavior of the basic nuclear proteins closely paralleled that of DNA. Thus, alterations of DNA levels in tumor tissues were accompanied by quantitatively similar changes in histone levels to maintain the same Feulgen/fast green ratios found in homologous normal tissues.

INTRODUCTION

For the most part, studies on histones or protamines have been restricted to animal tissues (18, 19, 22, 27, 41, 49, 57), although basic proteins are known to occur in several tissues of higher plants. For instance, the presence of a nucleohistone in wheat germ was noted by Mirsky and Pollister (40), and a basic protein characterized as a protamine has been reported for spores of *Lycopodium* (20). From cytochemical evidence, Kaufmann *et al.*, (34) suggested the association of a histone type protein with both the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) of onion root nuclei. More recently, Alfert and Bern (cited in 1) found a 1:1 ratio in comparing DNA and histone levels in intermitotic nuclei from growing onion root tips, although Bryan (17) earlier reported widely differing DNA/histone ratios in several tissues of the spider lily, *Tradescantia*.

In the past the basic protein fractions of individual nuclei have been difficult to study cytochemically because of a lack of specificity in the methods available (53 for review). Following the recent introduction of high pH acid dye binding as a selective stain for the basic proteins of fixed tissue nuclei (6), there have been several studies on animal cells (1, 2, 4, 9–12, 14, 16, 43, 44). In salmon testis (3) and embryonic mouse liver (15) histones showed a topographic distribution identical to that found for DNA. Histone levels also showed quantitative correlation with DNA content in a number of cell types (1, 5, 12, 15, 43). For example, although there were marked changes in total nuclear protein levels, histones demonstrated a constancy similar to that of DNA in rat thyroid and mouse kidney nuclei under diverse experimental conditions (4, 5, 12, 56). Similarly in lymphocytes of rat and man, the mean nuclear content of histone was closely correlated with DNA content, although in these and other tissue types, total protein showed no dependency on DNA content of the nucleus, but varied with nuclear volume (43, 44). The cytochemical findings to date are in general agree-

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ment with results obtained previously from direct chemical analyses, which show a fairly constant ratio of DNA to histone in a number of somatic vertebrate tissues (22, 41, 56 *a*, 57).

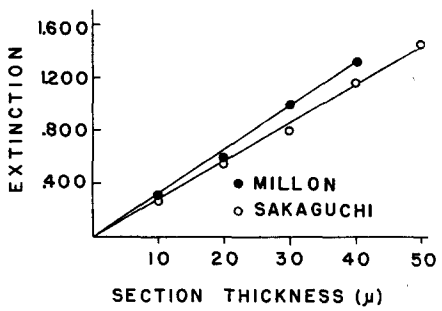
It was the purpose of this study to determine by microphotometry the quantitative relationships between DNA and histones in plant tissues. *Tradescantia paludosa* provided a good cytological object in which to estimate the basic protein content of nuclei in both meristematic and nondividing tissues, during gametogenesis, and embryogeny. In addition, several stages of crown-gall tumor growth in bean (*Vicia faba*) were used to study DNA/histone ratios during pathological cell duplication and differentiation.

Materials and Methods

Tissues were fixed in 10 per cent neutral formalin for 3 hours, washed overnight in running water, dehydrated, and paraffin embedded. Details of tumor induction in bean stems have been given elsewhere (47). Sections of appropriate thickness for a particular stage or tissue type were mounted on individual slides, and all slides in any one experiment were then treated simultaneously through the hydrolysis and staining procedures outlined by Alfert and Geschwind (6). To avoid a temperature drop in the 90°C. trichloroacetic acid (TCA) bath, slides were immersed for 1 minute in distilled water at 90°C., and then transferred immediately into the hot acid. Following 3 rinses in 70 per cent ethanol, slides were given 2 changes of distilled water adjusted to pH 8.1, before being placed in a freshly prepared aqueous solution of 0.1 per cent fast green at pH 8.1. Specificity of staining was routinely checked by the use of unextracted control preparations, which were otherwise processed with treated slides. In no case was fast green staining at pH 8.1 observed without prior nucleic acid removal. Careful matching of tissues by graded refractive index liquids showed least scattering of light by anther sections at n_D 1.572, and by embryo sac contents at n_D 1.564. Bean tissues were matched at n_D 1.568.

In several cases the Feulgen reaction was used on sections adjacent to those which had been stained for histones with fast green. A standard Feulgen procedure was followed, using 14 minutes of hydrolysis in N HCl at 60°C.

In order to minimize distributional and stray light errors (54), microphotometric determinations of fast green staining in individual nuclei were made at 580 mμ. Both *Tradescantia* and bean nuclei gave extinctions above 1.0 at 640 mμ, the absorption maximum of bound fast green. Light was isolated by a Beckman model DU spectrophotometer. Histone content has been expressed in photometric units. The total amount



TEXT-FIG. 1. Lambert law relations for microphotometric determinations of chromophore intensity with the Millon reaction for tyrosine and the Sakaguchi reaction for arginine applied to formalin-fixed egg albumen. Each point is the mean of 10 measurements. Section thickness was determined directly at a magnification of 1000 on folds adjacent to measured areas.

(M) of fast green staining per nucleus was calculated from $M = (EC^2/F)$, in which E is the extinction, or optical density, obtained from a "plug" sample with radius C through the nucleus, and F is that fraction of the total volume of the nucleus included in the core sample. The constant π has been omitted. Additional details of measuring technique and computation have been recently discussed elsewhere (54). The validity of microphotometric determination of alkaline fast green dye binding has been previously established by Bloch and Godman (15).

A modified Sakaguchi reaction for arginine and the Millon reaction for tyrosine were performed on anther and ovary sections from several slides which had previously been stained and measured as described above. The arginine reaction used has been modified slightly from that of Warren and McManus (58). Following a 3 minute incubation at 25°C. in 0.5 per cent 8-hydroxyquinoline (Eastman Kodak) in absolute ethanol, preparations were developed for 30 to 45 seconds in a 1:1:1 mixture of 10 per cent NaOH, commercial "clorox", and distilled water. The latter step was carried out at 0 to 1°C. in an ice bath. Preparations were immediately rinsed for 3 to 5 seconds with ice cold distilled water, and then passed rapidly through 3 changes of tertiary butyl alcohol, each change from 5 to 10 seconds. Clearing in xylol was followed by mounting in refractive index oil. The chromophore so developed was stable for at least 3 weeks. The Millon reaction used was modified from the method originally given by Pollister and Ris (45). The reaction here was performed in two steps. In step I the slides were treated with 5 per cent mercuric acetate in 1.8 M TCA at 40°C. for 10 minutes. For step II, the slides were transferred to another bath at 30°C. with the same concentrations of TCA and mercuric acetate plus 0.1 per cent $NaNO_2$ for 1 hour. Following three 5 minute rinses in 70 per

TABLE I

*Comparison of Deoxyribonuclease (DNase) and Trichloroacetic Acid (TCA) Removal of DNA on Alkaline Fast Green Staining of Tradescantia Nuclei**

Series	Cell type	DNase removal					TCA removal				
		Nuclear volume	Fast green-histone				Nuclear volume	Fast green-histone			
			Mean	S.E.	*n*	Ratio		Mean	S.E.	*n*	Ratio
		μ^3					μ^3				
I	Pollen mother cell	553	10.90	± 0.29	12	3.67	479	9.86	± 0.13	12	3.72
	Microspore	73	2.97	± 0.11	12		70	2.65	± 0.05	12	
II	Zygote (=fertilization)	335	3.49	—	4	1.89	348	4.84	± 0.10	20	1.93
	Egg	211	1.85	—	4		231	2.52	± 0.17	6	

* Amounts expressed in photometric units. The two series of experiments were performed at different times, and hence the values for Series I cannot be compared directly with those for Series II. Variability of staining from one experiment to another makes it essential that tissues to be compared be treated simultaneously through the extraction and staining procedures (see also 14). *n*, number of measurements.

cent ethanol, sections were dehydrated in 95 per cent and absolute ethanol, cleared in xylol, and mounted in refractive index oil. The Millon chromophore was stable for at least 3 months. Slides which had first been stained by the fast green procedure could subsequently be restained for quantitative measurement of the Millon or Sakaguchi reactions. Removal of fast green staining during the course of the protein reactions was checked by absorption curve analysis. There were no significant alterations in curve shape, height, nor in position of absorption maxima of the protein chromophores in comparisons of curves from previously unstained sections with those from sections which had first been stained with alkaline fast green. A linear relationship was found between optical density or extinction and section thickness, using formalin-fixed egg albumen as a test protein for the Millon and the Sakaguchi reactions (Text-fig. 1). Specificity and problems of quantitation have been previously discussed by McLeish *et al.* (36) for the cytochemical arginine reaction, and by Rasch and Swift (46) for the cytochemical Millon reaction. Stained specimens for photometry were mounted in matching refractive index oils (n_D 1.540 for arginine preparations, and n_D 1.560 for Millon preparations). Estimates of amounts of arginine per nucleus were made at 510 mμ, those for tyrosine at 500 mμ, the visible light absorption maxima of the developed chromophores. Estimates given are for comparative purposes only, and no attempt has been made to relate the values to absolute protein or amino acid content. Measurements were made on whole nuclei, and appropriate corrections for the surrounding matrix of absorbing cytoplasm have been applied (54).

Several experiments were performed to test the specificity of fast green staining by plant histones. As previously noted by Bloch and Godman (16), the usual operational definition of histones (and protamines) by virtue of their acid solubility cannot be applied to tissue sections following formalin fixation. After fixation in absolute methanol, however, the selective chromatin staining of plant nuclei with alkaline fast green after removal of nucleic acids can be virtually eliminated by additional extraction of tissue sections before staining in M NaCl at pH 3.8 for 4 hours at 25°C. (Figs. 1 and 2), and can be completely abolished by extraction in 0.01 N HCl for 4 hours at 25°C. or within 8 to 10 hours at 4°C. Similar loss of nuclear stainability was obtained for tissues stained by the Sakaguchi reaction for arginine (Figs. 3 and 4). These two standard extraction techniques (21, 22) define the histone nature of nuclear protein staining by alkaline fast green in these plant tissues.

In another series of experiments, total nucleic acid extraction with hot TCA was compared with the effect of DNA removal only. These two methods should give rise to different kinds of staining artifacts (3, 7). Sections of formalin-fixed tissues were pretreated in distilled water at 90°C. for 10 minutes (3, 53) before incubation for 1 hour at 27°C. in a fresh solution of purified deoxyribonuclease (Worthington, 3X crystallized, 0.5 mg./ml. in 0.003 M $MgSO_4$, pH 6.5). Absence of Feulgen staining after nuclease treatment demonstrated the successful removal of DNA. Selective chromatin staining with alkaline fast green was found in tissues of corn and lily, as well as in *Tradescantia* and bean, irrespective of the method of nucleic acid removal (Table I, Figs. 11 and 12). Increasing dye content of the fast green solution from the recommended 0.1 to 0.5 per cent also did not affect the chromatin specificity of histone staining, and increased the total amount of dye bound per nucleus by only

15 to 20 per cent. Generally similar results were obtained with the acid dyes naphthol yellow S and orange G when used at pH 8.0 to 8.2.

Two methods can be used to block completely the selective nuclear staining normally found in plant tissues with alkaline fast green. The first entails extraction of sections with hot TCA, then treatment with 0.01 to 0.1 per cent DNA in 0.1 M NaCl, before staining with fast green at pH 8.1. Histone staining was abolished by this procedure and could be restored in the sections only by an additional hot TCA hydrolysis following the DNA treatment. The second method involves deamination of tissue protein by treating sections with $NaNO_2$ in an acetic acid–ethanol solution or by acetylation of amino groups with acetic anhydride (3, 11, 15). These treatments, either before or after nucleic acid extraction, abolished alkaline fast green dye binding and were irreversible. The inhibition of acid dye binding observed is to be expected, since addition of nucleic acids to tissue sections, or the removal of basic groups from tissue proteins would result in a net negative charge on the substrate (53). These data may also be taken to indicate that the Alfert-Geschwind reaction (fast green staining at pH 8) primarily reflects the over-all balance of acid to basic groups, *i.e.*, the net positive charge of the protein. Therefore, histone is here operationally defined as that arginine-rich nuclear protein fraction which (1) is extracted from methanol fixed tissues with 0.01 N HCl or M NaCl at pH 3.8, and (2) is revealed in formalin-fixed tissues by alkaline fast green staining after removal of nucleic acids by hot TCA or by deoxyribonuclease extraction following hot water pretreatment of tissue sections.

As emphasized by Alfert (1, 4), DNA determinations are important for comparative studies of the protein fractions of interphase nuclei, because such values provide a quantitative definition of chromosome or chromatid number, and thus give a base line for evaluating changes in protein content. The nuclear class or "C" value for each cell type in Tables I to IV are those reported for DNA content of nuclei of *Tradescantia* from several laboratories (42, 52, 59). In Tables V and VI DNA amounts were estimated directly by the usual photometric techniques (54) applied to Feulgen-stained sections adjacent to those on which histone determinations were made.

RESULTS AND OBSERVATIONS

I. Normal Tissues

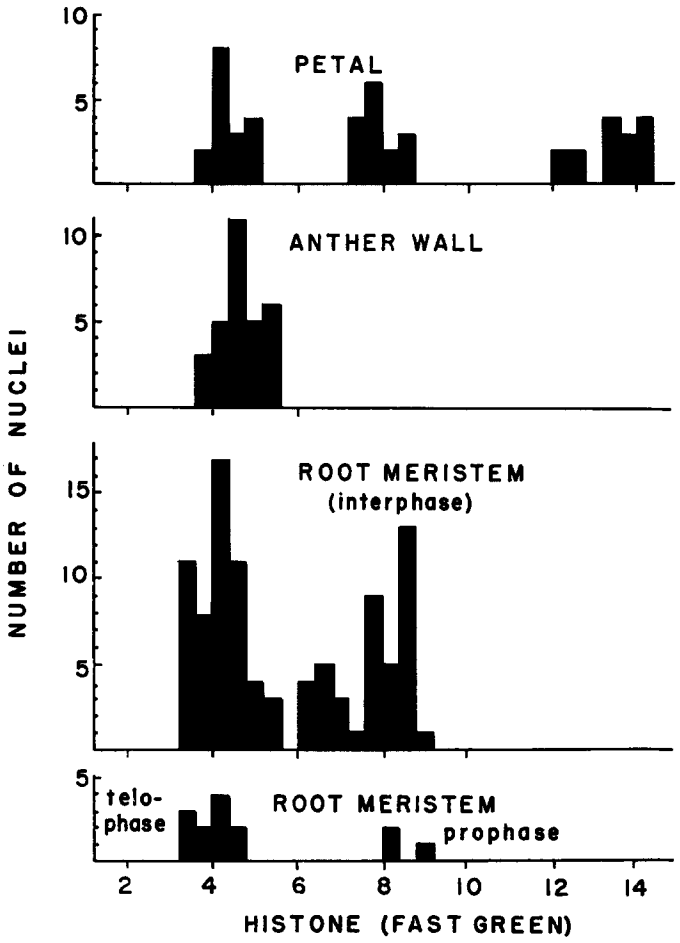
The behavior of histones in both dividing and non-dividing somatic tissues of *Tradescantia* is illustrated in Text-fig. 2. In leaf, petal, and anther wall, where a 1:2:4 series of DNA-Feulgen values has been found (52), amounts of fast green–histone also approximated such a seriation (Table II). As demonstrated in the histograms, no intermediate histone values were found in these tissues.

Histograms of histone values from meristematic integuments of young ovaries or in growing root tips, on the other hand, showed a grouping of values for prophase nuclei (4C) at twice the telophase amount (2C), and a spread of interclass values between the 2C and 4C amounts for interphase nuclei (Text-fig. 2 and Table II).

Cytochemical changes in proliferating tissues may be related to time by considering the frequency of nuclear volume classes to be proportional to percentage of elapsed interphase time (28, and Woodard, unpublished). Histone values for *Tradescantia* root nuclei are plotted against such a relative time scale in Text-fig. 3 (bottom). The curve for histone accumulation in intermitotic nuclei did not follow that for total protein increase, insofar as the latter can be judged from the curve for cumulative volume increase plotted against relative interphase time (Text-fig. 3, top).[1] For example, during the first third of interphase histone amounts remained essentially unchanged although there was a 65 per cent increase in nuclear volume during this period. Nuclear histone content in these root meristems seems clearly independent of changes in nuclear volume, and presumably then from changes in amounts of other nuclear protein fractions. It deserves to be emphasized that the idealized curve for histone synthesis during interphase is like that previously reported for DNA synthesis in this and other root meristems (47, 53).

The behavior of histones during gamete formation and embryogeny is illustrated in Text-figs. 4 and 5, based on the data of Table II. During microsporogenesis the amount of fast green staining by meiotic pachytene nuclei (4C) was four times the basic amount (C) found for haploid microspores or gametes. Successive halving of the 4C amount occurred during each of the two meiotic divisions to yield the haploid (C) tetrad nucleus. An increase to the 2C amount prior to microspore prophase preceded the division which formed a two-nucleate microgametophyte. Increase to the 2C level occurred very early in the generative nucleus of the pollen grain, although the histone content of the vegetative nucleus remained

[1] In bean root meristems the curves for cumulative nuclear volume increase and for net accumulation of total nuclear protein during interphase coincide (Woodard, unpublished).



TEXT-FIG. 2. Histograms of relative amounts of histone (fast green) per nucleus in petal and anther wall tissues, and in the root meristem of *Tradescantia paludosa*.

unchanged up to anthesis. The histone content of egg nuclei (C) was approximately equal to that found for microgamete or sperm nuclei. As indicated in Table II and in Text-fig. 5, like DNA, the histone contribution by egg and microgamete at the time of fertilization and in the 2C zygote was approximately equal. These results were independent of the method of DNA removal (Table I, Figs. 11 to 13).

Nuclei from young embryos showed a bimodal distribution of histone amounts with several values between the 2C level of telophase nuclei and the 4C level of prophase nuclei (Text-fig. 5). Endosperm nuclei, which result from the triple fusion of two haploid polar nuclei and a microgamete nucleus, showed a similar bimodal distribution, but with means at 3 and 6 times the histone content of gamete nuclei (Text-fig. 5 and Table II). The absence of interclass values for nuclei of embryos from shed seeds is consistent with the lack of mitosis at this stage of embryo maturation (Fig. 14).

Several different techniques were used to investigate possible qualitative changes in histone composition during various stages of gametogenesis. Estimates of nuclear arginine content during microsporogenesis and for several differentiated somatic tissues correlated with the step-wise alterations in histone content found with alkaline fast green staining (Table III). The elevated arginine content of egg nuclei is an exception to this pattern and probably reflects a general increase in total protein as shown from the estimates of nuclear tyrosine content (Table

TABLE II

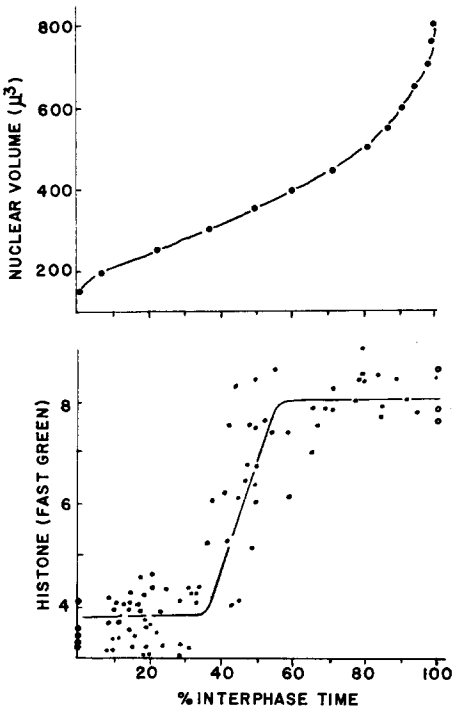
Relative Amounts of Histone (Fast Green) in Individual Nuclei of Tradescantia paludosa

Cell type	Nuclear volume	Fast green–histone			DNA class ("C" value)
		Mean	S.E.	*n*	
	μ^3				
Sporogenous					
Pollen mother cell*	736	9.21	0.27	34	4
Dyad	373	4.23	0.32	15	2
Quartet (tetrad)	139	2.09	0.07	27	1
Microspore I* (early)	97	2.12	0.05	27	1
Microspore II	164	2.16	0.18	27	1
Microspore III	306	3.08	0.55	52	1→2
Microspore IV* (late)	472	4.72	0.22	25	2
Microspore prophase*	582	4.69	0.10	12	2
Microgametophyte					
Early pollen grain					
Vegetative	297	2.32	0.05	50	1
Generative*	255	3.96	0.36	55	1→2
Late pollen grain					
Vegetative*	311	2.19	0.21	12	1
Microgamete‡ (shed sperm)	50	1.77	—	5	1
Megagametophyte					
Egg or synergid‡	231	2.11	0.13	7	1
Fertilization‡	348	4.11	0.10	20	2
Embryo					
1-cell zygote‡	751	5.42	—	28	2→4
Endosperm‡	881	6.14	0.20	15	3
	1995	10.84	0.47	14	6
Mature embryo‡ (shed seed)	289	4.60	—	3	2
	427	7.72	0.43	25	4
Somatic					
Leaf	178	4.05	0.06	10	2
	218	7.97	0.16	20	4
Anther wall§	229	4.46	0.14	30	2
Petal§	183	4.17	0.16	17	2
	258	7.32	0.25	15	4
	521	13.22	0.31	15	8
Ovary integuments					
Telophase	145	4.85	0.11	12	2
Interphase (early)	208	4.57	0.09	15	2
Prophase	559	9.71	0.36	15	4
Root meristem§					
Telophase	198	3.81	0.19	8	2
Interphase	400	4.86	—	95	2→4
Prophase	747	8.09	—	3	4

* Data plotted in Text-fig. 4.
‡ Data plotted in Text-fig. 5.
§ Data plotted in Text-fig. 2.

IV). In view of the increased tyrosine content of egg nuclei, certainly a large part of the increase in arginine content observed could be ascribed to change in amount or composition of one or more non-histone nuclear protein fractions. Similar reasoning can be applied to the data for arginine and tyrosine content of vegetative nuclei of mature pollen grains.

Alkaline fast green staining following nuclease removal of DNA should present a picture of total



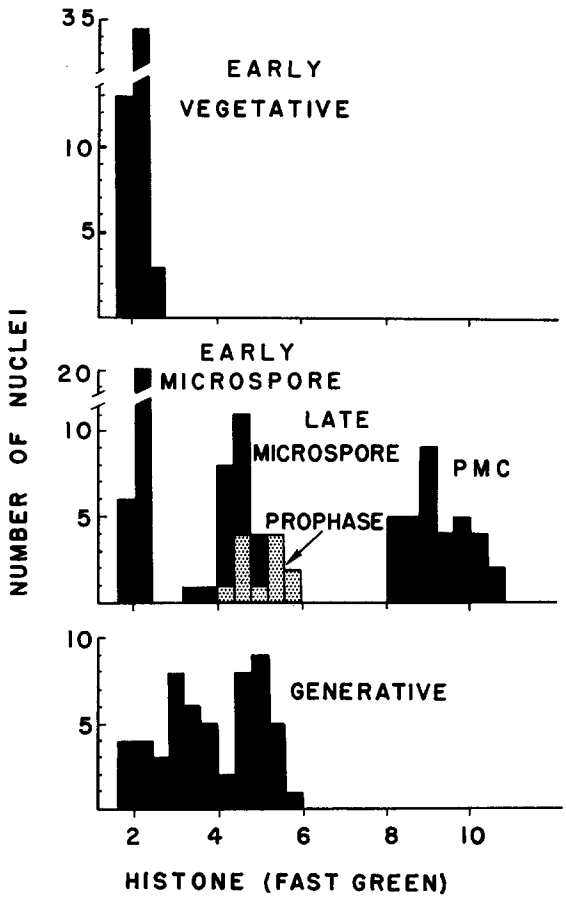
TEXT-FIG. 3. Nuclear volume increase (top) and nuclear histone increase (bottom) plotted against percentage interphase time for *Tradescantia* root tip nuclei. Open circles at left represent telophase nuclei; those at right are prophase nuclei.

basic protein, whereas hot TCA extraction would be expected to remove protamines (3, 6), if these simpler and more basic proteins were formed during plant gametogenesis. To test this possibility, a series of slides of several anther stages and of ovaries at various times before and after pollination was prepared. One member of a pair of slides was extracted with deoxyribonuclease, the other member with hot TCA, prior to being stained simultaneously in fast green at pH 8.1. The histone content of egg nuclei following either extraction method approximated that found for haploid microspore nuclei (Table I), or that found for microgamete nuclei (see Figs. 11 and 12). Microscopic inspection of embryo sacs collected at 14 hours after pollination also showed that the two newly formed microgametes stained heavily with alkaline fast green, irrespective of the method of DNA removal. Furthermore, as indicated in Tables I and II (and Figs. 11 to 13) the contribution by egg and sperm and fertilization was approximately equal, whether the DNA was removed by nuclease or by hot TCA. The 2 per cent difference in ratios of histone content of zygote nucleus/egg nucleus (Table I) indicated no significant loss of basic protein by microgamete nuclei during acid extraction. Similar comparisons of nuclease or hot acid extraction prior to fast green staining for the mature, three-nucleate pollen grain of corn demonstrated that here also the two newly formed microgamete nuclei stained equally well with either method of nucleic acid removal.

From these several lines of evidence we conclude (1) there are no marked qualitative changes in histone composition of plant nuclei during gametogenesis, and (2) the functional sperm nucleus of these higher plants contains a basic protein of the histone type.

Although the above experiments were not designed to investigate the total protein content of nuclei of the microgametophyte, several additional observations on the vegetative nucleus of young and maturing pollen grains seem worthy of note. Estimates of nuclear arginine and tyrosine levels showed a 150 per cent increase in arginine content and a 110 per cent increase in tyrosine content when vegetative nuclei were compared to quartet or young microspore nuclei (Tables III and IV). Since histone content (alkaline fast green) was unchanged during this period of development (Table II), the data are interpreted to show a significant change in the non-histone protein fractions of these nuclei. This major alteration in nuclear protein content or composition may well be correlated with the increased amounts of RNA and protein found in the cytoplasm of maturing pollen grains (60). It is worth noting in this connection that the cytoplasm of even the late microspore is essentially negative for arginine, but coincident to the increase in arginine content of the microgametophyte nuclei, concentration of arginine in the cytoplasm increased about 100 per cent. These changes, in general, paralleled increases in tyrosine concentration for the two cell types. For the microspore, extinctions of the Millon chromophore per μ^3 of cytoplasm averaged 0.014 (λ = 500 mμ), but for the cytoplasm of the pollen grain extinctions averaged 0.032 per μ^3. These changes in concentration occurred during a period of considerable volume increase of the microgametophyte, and therefore indicate a net increase in total protein content of several hundred per cent.

Another pecularity of microgametophyte development was the consistent difference in staining behavior of generative and vegetative nuclei following nuclease extraction of DNA. Although the amount of alkaline fast green staining by vegetative nuclei was unchanged in young, mid, or mature pollen grains following hot TCA extraction, after nuclease treatment of sections

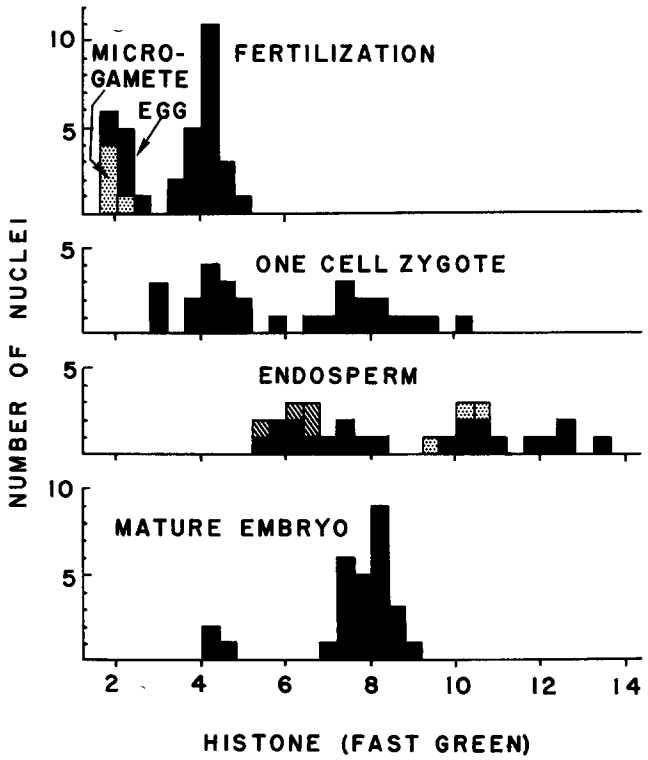


TEXT-FIG. 4. Histograms of relative amounts of histone (fast green) per nucleus during stages of microsporogenesis and pollen grain formation in *Tradescantia*. (*PMC* = pollen mother cell).

intensity of fast green staining by vegetative nuclei decreased with progressive stages of pollen grain development (Figs. 5 through 8). Staining of generative nuclei was the same with both methods of DNA removal (Figs. 8 and 10). Standard Feulgen procedures, applied to sections previously treated with nuclease and stained with fast green, demonstrated that incomplete DNA removal was responsible for the decreased fast green dye binding observed in older vegetative nuclei (compare Figs. 8 and 9). With methanol or acetic acid–ethanol fixation, complete removal of DNA from both generative and vegetative nuclei was easily obtained with the usual nuclease treatment. Following formalin fixation, however, the susceptibility or availability of the DNA in older vegetative nuclei to action by deoxyribonuclease seemed markedly different from that found for other somatic or sporogenous nuclei. Conceivably this nuclease-resistant DNA could indicate the appearance of a particular DNA-protein complex, perhaps that associated with active protein synthesis (39). In this connection, Alfert (3) suggested from studies of salmon testes that the susceptibility of nuclei to deoxyribonuclease after formalin fixation occurs indirectly by way of a non-histone protein component lacking in salmon sperm nuclei.

II. Pathological Tissues

The quantitative behavior of basic proteins of tumor nuclei was studied by comparing DNA/histone ratios from normal tissues with those from crown-gall tumors of bean. Crown-gall is a neoplastic growth of higher plants induced by inoculation of susceptible host tissues with virulent bacteria. The complex cytological and cytochemical changes accompanying tumor genesis and growth in bean are reported in detail elsewhere (47). For the present study, one question is of primary importance: how does the histone



TEXT-FIG. 5. Histograms of relative amounts of histone (fast green) per nucleus during formation of mature gametes, at time of fertilization, and during embryo growth and maturation in *Tradescantia*. In the histogram of endosperm nuclei, barred areas at left represent telophase nuclei; dotted areas at right are prophase nuclei.

content of tumor nuclei compare with that of normal nuclei? The data in Table V are estimates of the mean amount of DNA-Feulgen or of fast green–histone per diploid (2C) nucleus in cells from normal unwounded bean stem, from wound repair tissues, and from stem tissues at varying times after inoculation with tumor-inducing bacteria. It is clear from the data that Feulgen/fast green ratios from all tissues were essentially the same.

As illustrated in Figs. 15 and 16, if means of all nuclei, irrespective of DNA class, are included in comparisons of histone levels in normal and tumor tissues of bean, tumor nuclei *on the average* have a significantly higher histone content than do normal nuclei. This behavior of histone is entirely consistent with the behavior of DNA in the system, and is, in part, a reflection of chromatin duplication accompanying mitosis during early tumor induction. In part, it is also a consequence of the progressive accumulation of highly polyploid or polytene nuclei during later tumor growth (47).

Similar observations have been made on animal tissues (Rasch, unpublished). Feulgen/fast green ratios for two normal mouse cell types, lymphocytes and kidney, were compared with those for the tumor cells of a transplantable melanoma (Cloudman S-91 in dba mice). The DNA content of melanoma nuclei was approximately twice that found for normal somatic nuclei. Since, however, there was also approximately twice as much fast green–histone in melanoma nuclei, the Feulgen/fast green ratio for the tumor population did not differ significantly from those for lymphocyte or kidney nuclei.

The data from both plant and animal tumor nuclei show that (1) no significant alteration in DNA/histone ratios occurred during pathological cell duplication or differentiation, and (2) an increased DNA content of tumor nuclei, associated with chromatin duplication and/or aneuploidy, was accompanied by quantitatively similar increases in histone content.

TABLE III

Estimates of Total Arginine (Sakaguchi) in Individual Nuclei of Tradescantia paludosa

Cell type	Nuclear volume	Arginine content			DNA class
		Mean	S.E.	*n*	
	μ^3				
Sporogenous					
Pollen mother cell	537	2.46	0.07	10	4
Dyad	344	1.33	0.10	12	2
Quartet (tetrad)	93	0.68	0.04	12	1
Microspore I	75	0.77	0.04	12	1
Microspore II	108	0.77	0.06	12	1
Microspore IV	449	1.34	0.05	18	2
Microgametophyte					
Mid-pollen grain					
Vegetative	189	1.70	0.13	10	1
Generative	155	1.63	0.09	15	2
Megagametophyte					
Egg	267	1.33	—	6	1
Somatic					
Anther wall	208	2.16	0.06	27	2
Petal	173	1.32	0.06	10	2
	338	2.27	0.08	9	4
	588	3.67	—	2	8

III. DNA/Histone Ratios

Although the relative amount of histone per nucleus varied widely from species to species, these basic proteins occurred in a remarkably constant proportion to DNA in a variety of plant cells. As shown in Table VI, the basic protein content of nuclei from four different families followed quantitatively their DNA content so that Feulgen/fast green ratios were essentially the same.[2] Thus in these rather diverse groups with widely varying DNA levels there appeared to be an identical amount of histone for every unit of DNA.

[2] The staining intensities given in Table VI are in photometric units and obviously do not in themselves indicate absolute amounts of DNA or histone in the tissues examined, but are for comparative purposes only. Estimates of actual DNA content per diploid (2C) nucleus, however, have been made for these several species by calibration of the plant Feulgen measurements with Feulgen-stained rat liver or kidney nuclei as a known standard of approximately 6×10^{-12} gm. DNA per 2C nucleus (see 47 for method applied to bean tissues). Assuming this calibration to be valid, diploid lily nuclei contain about 100×10^{-12} gm. DNA; *Tradescantia* nuclei about 52×10^{-12} gm. DNA; onion nuclei about 40×10^{-12} gm. DNA; bean nuclei about 18×10^{-12} gm. DNA; and diploid corn nuclei about 4×10^{-12} gm. DNA.

DISCUSSION

The stable quantitative behavior of histones and DNA in plant tissues is emphasized by comparisons with fluctuating levels of other nuclear components in cells of varying physiological states. For example, during microgametophyte development in *Tradescantia* (Tables III and IV) or during crown-gall tumor induction in bean (47) significant increases in amounts of total nuclear protein were correlated with marked changes in nuclear volume, but were independent of DNA content. DNA/histone ratios in these tissues remained unchanged, but as a consequence of nuclear enlargement, histone concentrations were significantly lowered in nuclei with high levels of non-histone protein. Findings for plant tissues thus agree with recent cytochemical studies of histone levels in animal tissues under varying conditions of physiological stress (1–6, 12). The stable DNA/histone ratios for plant and animal tissues determined cytochemically are also consistent with results obtained recently from direct chemical analyses which indicated no significant differences in histone content or composition between normal and pycnotic calf kidney nuclei (56). This stable behavior of histone might be expected also from studies of isotope incorporation which have shown that histones, like DNA, are relatively inert when compared to the metabolically active protein fractions of the nucleus (8, 21, 31, 39).

Cytochemical techniques for staining histones have as yet given little information as to the heterogeneity in composition of these basic proteins from any one cell type, as described first by the Stedmans (49, 50) and later by other workers (8, 18, 19, 22, 26, 29, 30, 35, 55). The composition of plant histones, for example during gametogenesis, seems relatively uniform, at least with regard to arginine and lysine content. (As discussed by Alfert and coworkers (1, 6) fast green dye binding at pH 8 or above is largely a function of arginine and lysine residues and the net positive charge they impart to the substrate after removal of nucleic acids. Therefore, alterations in Feulgen/fast green ratios may reflect intrinsic differences among basic proteins (3, 6, 11), changes in the chemical union between histone and DNA (2,

TABLE IV

Relative Amounts of Total Protein (Millon) in Individual Nuclei of Tradescantia paludosa

Cell type	Nuclear volume	Total protein (Millon)			DNA class
		Mean	S.E.	*n*	
	μ^3				
Sporogenous					
Quartet	120	1.07	0.19	8	1
Microspore I	75	0.97	0.06	15	1
Microgametophyte					
Pollen grain					
Vegetative	221	2.17	0.20	10	1
Generative	172	2.26	—	5	2
Megametophyte					
Egg-synergid	279	1.68	—	6	1
Somatic					
Anther wall	398	3.18	0.27	10	4

TABLE V

*DNA-Feulgen and Fast Green–Histone Amounts in Normal Bean Cells, in Wound Repair Tissue, and during Crown-Gall Tumor Formation in Bean Stem Tissue**

Tissue type	Nuclear volume	DNA-Feulgen			Fast green–histone			Ratio: Feulgen/ fast green
		Mean	S.E.	*n*	Mean	S.E.	*n*	
	μ^3							
Normal stem	88	1.95	0.08	16	1.62	0.08	15	1.20
Wound repair cells	166	1.97	0.02	12	1.84	0.06	12	1.07
Crown-gall tumor, (days after inoculation)								
2 days	221	1.90	0.13	25	1.67	0.06	15	1.14
3 days	158	1.88	0.07	12	1.67	0.06	21	1.12
5 days	167	1.93	0.12	17	1.64	0.08	15	1.18
9 days	151	1.77	0.08	15	1.50	0.07	17	1.18
20 days	124	1.78	0.09	17	1.58	0.07	20	1.13
33 days	132	1.90	0.12	17	1.64	0.08	20	1.16

* Nuclei selected for comparison were in the same volume class as those nuclei known to be diploid (2C) by DNA-Feulgen measurements on the adjacent section. Feulgen determinations were made at 600 mμ; fast green estimates at 580 mμ.

9, 10), or competitive interference to dye binding by other nucleoprotein fractions (7, 14–16).) Uniform composition of plant histones is suggested also by recent microphotometric studies of nuclear arginine content by McLeish and Sherratt (37). These authors found Sakaguchi values which were directly proportional to polyploidy (DNA-Feulgen) in *Scilla* roots, in diploid, triploid, and tetraploid *Ranunculus* plants, and in diploid and tetraploid plants of *Tradescantia ohioensis*. These findings are consistent with the hypothesis that arginine residues are fixed to acidic groups of DNA as bonds between nucleic acid and protein moieties of the nucleoprotein (27, 33, 56 *a*, 57). DNA/histone ratios in *Tradescantia* have been discussed in one other microphotometric study (17). Histone estimates were, however, based on the difference in absorption of the Millon chromophore obtained for total protein and for acid-insoluble protein, and hence are not directly com-

TABLE VI

*DNA/Histone Ratios in Several Plant Species**

Species	Cell type	Nuclear volume	DNA-Feulgen			Fast green-histone			Ratio: Feulgen/ fast green
			Mean	S.E.	*n*	Mean	S.E.	*n*	
		μ^3							
Allium cepa	Root interphase	158	3.04	0.12	12	2.68	0.02	15	1.13
	Root telophase	145	2.92	0.08	15	2.65	0.07	20	1.10
Lilium henryi	Anther wall	316	9.19	0.49	15	8.82	0.34	14	1.04
	Quartets	252	5.02	0.11	15	4.83	0.17	15	1.16
Rheo discolor	Anther wall	78	2.14	0.05	12	1.83	0.08	12	1.17
Tradescantia paludosa	Anther wall	195	4.35	0.04	12	4.06	0.08	12	1.07
Vicia faba	Normal stem	88	1.95	0.02	15	1.62	0.08	12	1.20
Zea mays	Anther wall	23	0.38	0.01	12	0.34	0.03	12	1.11

* Sections of each species and tissue type were hydrolyzed and stained simultaneously for the Feulgen reaction. Adjacent sections were extracted with TCA at 90°C. for 15 minutes and then stained back to back in fast green at pH 8.1. For comparisons of Feulgen/fast green ratios, diploid or 2C nuclei were selected on the basis of DNA content in Feulgen preparations and on the basis of nuclear volume class in the fast green preparations. Feulgen measurements were made at 600 mμ; fast green measurements were at 580 mμ.

parable with the data on selective histone staining presented here.

Concomitant increases in amounts of DNA and histone during a period in interphase prior to cell division have suggested simultaneous duplication of a nucleohistone complex (1, 13–15). In the present study, the curve for histone synthesis in root tip interphase nuclei fits with this interpretation, as do the DNA/histone ratios for tumors and homologous normal tissues. Irvin *et al.* (32) have also suggested such an association of DNA and histone from comparisons of incorporation rates by these fractions in transplanted hepatomas and normal rat livers.

Relatively few papers deal with functional aspects of histones (4, 13, 14, 19, 25, 48, 49), despite a voluminous literature on analytical aspects of basic nuclear proteins (see 18, 19, 22, and 27 for reviews). Several biological roles for histone have been proposed. One hypothesis, that histones act as regulators of mitosis by their control of DNA synthesis, was first suggested by the Stedmans (48) from evidence of decreased histone levels in embryonic or tumor nuclei compared with differentiated adult tissues. Results were expressed as per cent lipide-free dry weight of nuclei isolated in acetic acid. As may be seen from Tables II and V, nuclei from embryonic or tumorous plant tissues characteristically have larger volumes, and coincidently a greater total protein content than do nuclei from normal, differentiated cells. Clearly then, although the *amount* of histone per 2C nucleus does not differ significantly when embryonic or tumor tissues are compared to normal adult plant tissues, the *concentration* of histones is much lower in growing tissues. Expression of tissue histone content as per cent dry weight (48, 50) in these and similar tissues will reflect alterations in total nuclear protein content and will not represent actual histone content per cell. In the several systems studied here, mean histone content per diploid nucleus was the same in tumorous or normal bean tissues, and in embryonic or differentiated lily tissues. Moreover, in tissues showing a geometric distribution of DNA values, such as petal or leaf, fast green estimates also showed a step-wise seriation of histone amounts. Evidence from the present study, therefore, does not support the hypothesis that histones act as regulators of mitosis merely by progressive accumulation in differentiated tissues to prevent DNA synthesis and thus inhibit mitosis (48, 50).

From the physical association of basic proteins

with DNA, at least as these fractions are obtained by the usual biochemical separation techniques (22–24, 27, 40, 41, 56), from the similarity in distribution seen cytologically (1, 3, 6, 14), and from the quantitative correlations of these two nuclear components to chromosome behavior in many plant and animal tissues, it is reasonable to presume that histones play an important role in the cell economy (see also 33, 38). As suggested by Stern (51) and Bloch (13, 14), for example, histones may act by "locking in" a specific DNA configuration which would genetically determine the pattern of cell modulation. Therefore, although histones may not necessarily be involved directly in transmission of genetic specificities, the possibility of a functional relationship between histones and DNA in the mechanism of gene action cannot be ignored.

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Explanation of Plates

Plate 140

Figs. 1 to 4. Extraction of basic proteins from sections of methanol-fixed *Tradescantia* anthers. × 490.

Fig. 1. Photomicrograph of control section, illustrating intensity and distribution of alkaline fast green staining of mid-microspore nuclei (*M*) and anther wall nuclei (*A*).

Fig. 2. Alternate section, showing greatly diminished fast green staining after extraction of histones with M sodium chloride at pH 3.8 for 4 hours at 25°C. Similar sections extracted with HCl show no staining whatsoever.

Fig. 3. Photomicrograph of control section indicating intensity of the chromophore developed in anther tissues by the Sakaguchi reaction for arginine. Mid-microspore nuclei (*M*) and anther wall nuclei (*A*).

Fig. 4. Alternate section demonstrating removal of arginine residues of nuclear proteins following extraction for 4 hours in M sodium chloride at pH 3.8 at 25°C.

Figs. 5 to 10. Differential removal of DNA from vegetative nuclei of formalin-fixed pollen grains by acid or nuclease treatment. × 2300.

Fig. 5. Alkaline fast green staining of microspore anaphase chromosomes after deoxyribonuclease extraction of DNA.

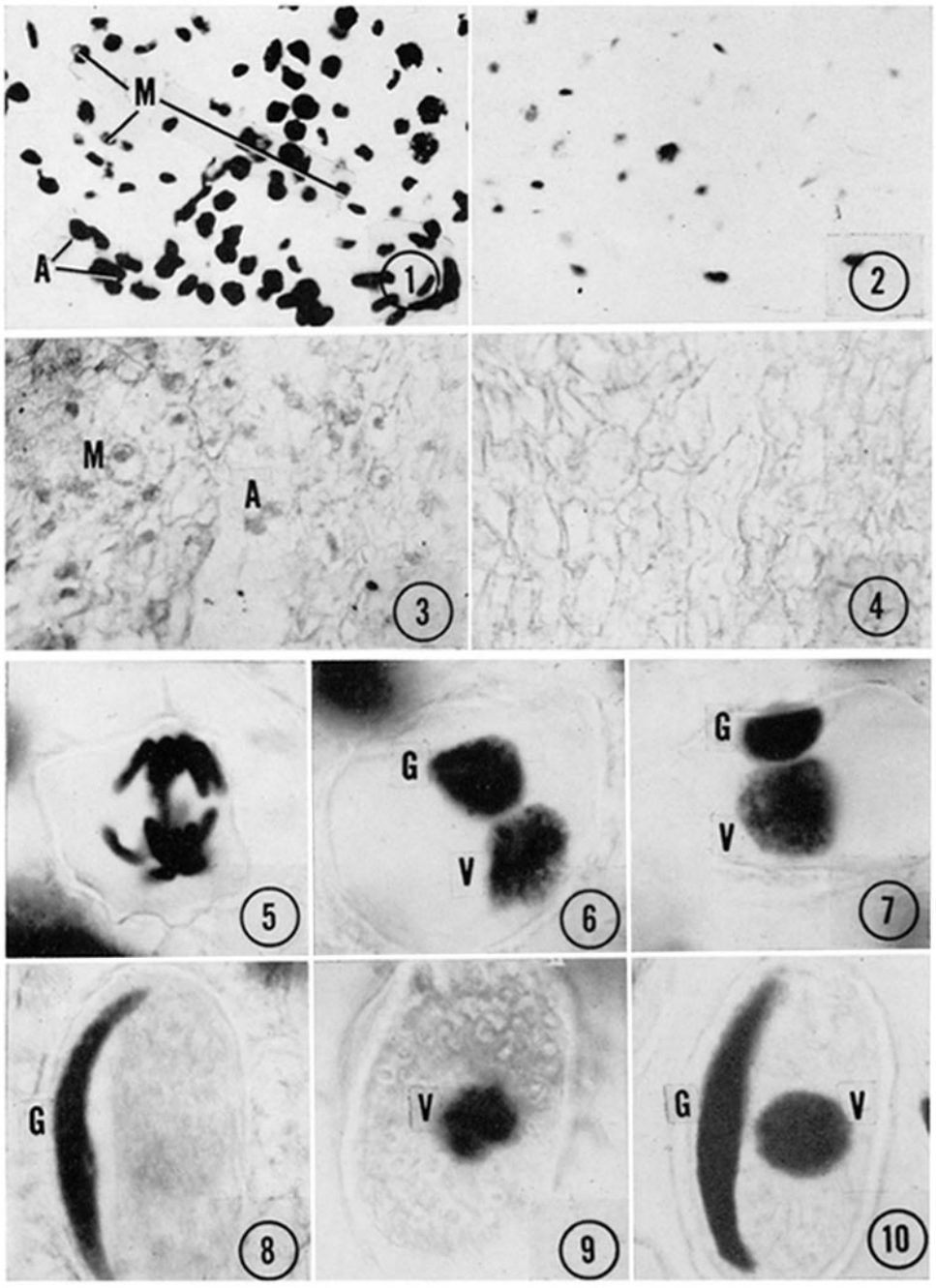
Fig. 6. Young pollen grain with vegetative (*V*) and generative (*G*) nuclei showing strong histone stainability with fast green after nuclease treatment.

Fig. 7. Early pollen grain. The generative nucleus is in its characteristic peripheral position and possesses twice the histone content of the centrally located vegetative nucleus. Nuclease extraction of DNA.

Fig. 8. Late pollen grain showing strong fast green staining in the curved generative nucleus (*G*). Note the lack of histone stainability by the vegetative nucleus here after nuclease removal of DNA.

Fig. 9. Late pollen grain of same section, now stained by the Feulgen reaction for DNA, to illustrate the differential removal of DNA by nuclease following formalin fixation. The vegetative nucleus (*V*) is strongly Feulgen-positive, while the generative nucleus is completely negative.

Fig. 10. Alternate section of late pollen grains from same anther as in Figs. 8 and 9. Both vegetative (*V*) and generative (*G*) nuclei show strong histone staining after hot TCA removal of nucleic acids.



(Rasch and Woodard: Basic proteins of plant nuclei)

PLATE 141

FIGS. 11 to 14. Alkaline fast green staining of *Tradescantia* nuclei during fertilization and embryo growth. × 1700.

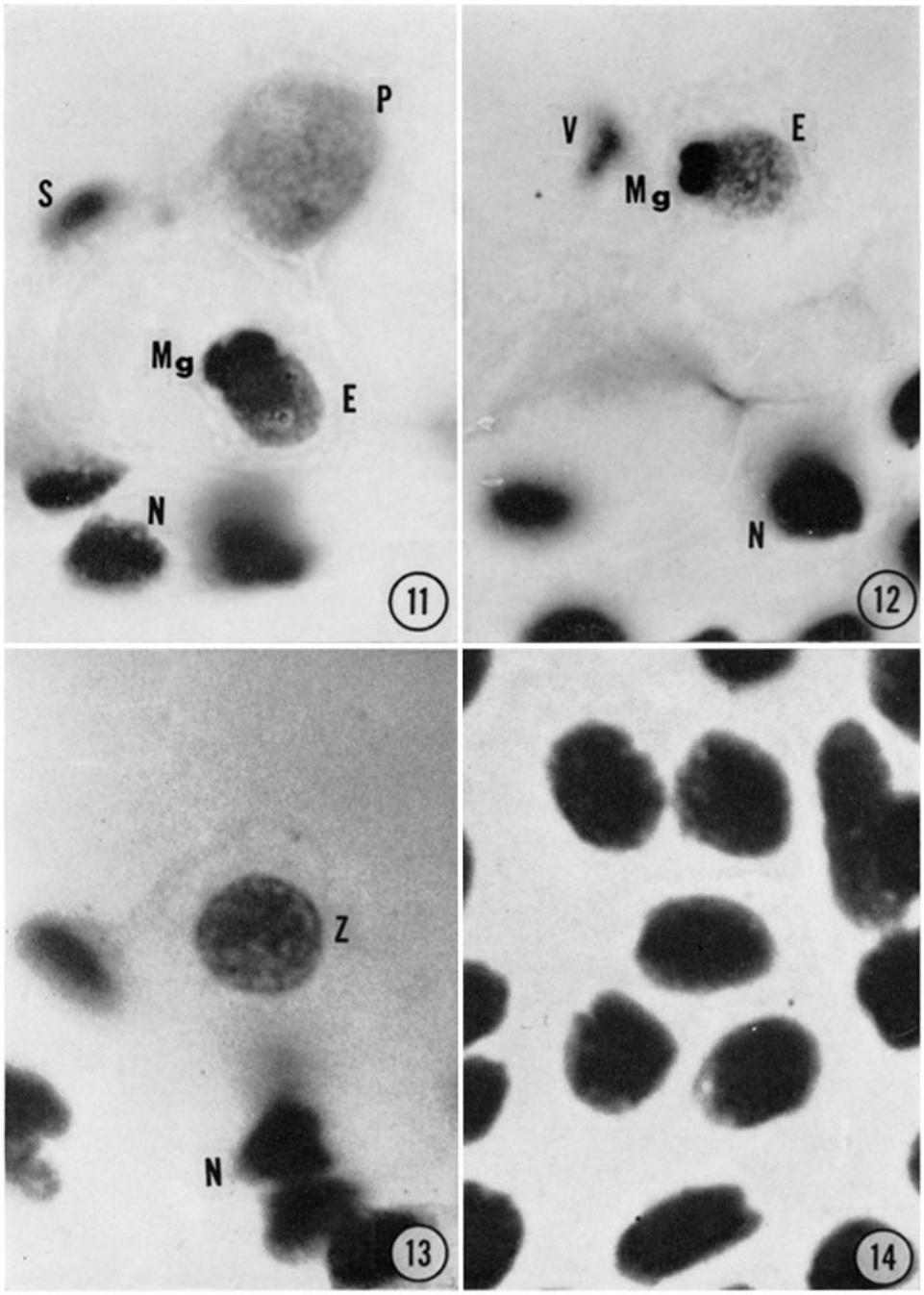
E, egg nucleus; *Mg*, microgamete (or sperm) nucleus; *N*, somatic nuclei of tissue surrounding embryo sac; *P*, polar nuclei; *S*, degenerating synergid nucleus; *V*, degenerating vegetative nucleus; *Z*, zygote or one-celled embryo.

FIG. 11. Embryo sac 20 hours after pollination, showing stainability of gametes following nuclease extraction of DNA.

FIG. 12. Embryo sac 19 hours after pollination, showing intensity of staining by gametes following removal of nucleic acids with TCA at 90°C. for 15 minutes.

FIG. 13. Zygote nucleus 48 hours after pollination. Hot TCA extraction of nucleic acids.

FIG. 14. Nuclei of mature embryo, dissected from shed seed. Nucleic acids extracted with hot TCA.



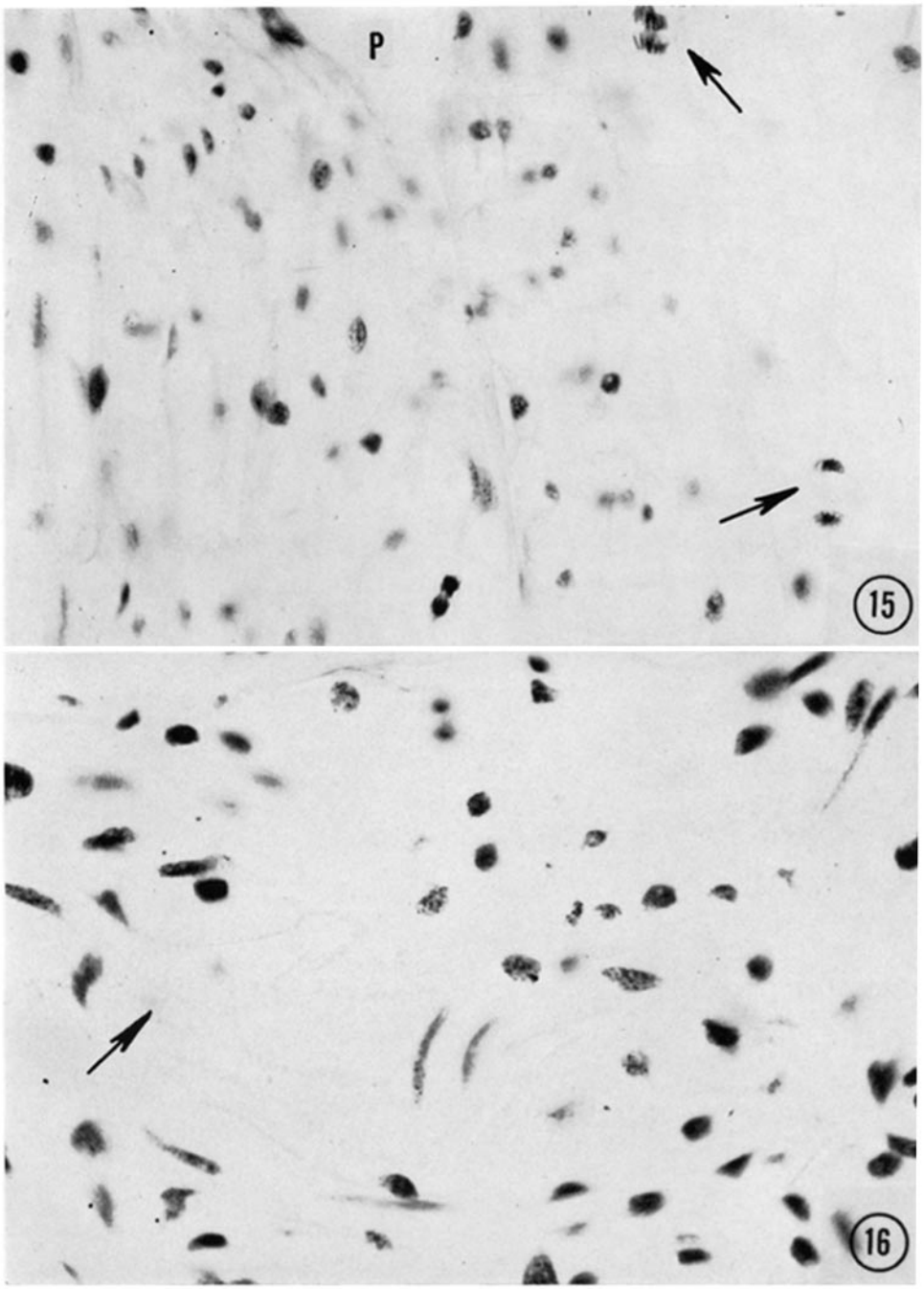
(Rasch and Woodard: Basic proteins of plant nuclei)

PLATE 142

FIGS. 15 and 16. Alkaline fast green staining of crown-gall tumor nuclei following hot TCA extraction of nucleic acids. Photomicrographs of longitudinal sections of broad bean stems. × 150.

FIG. 15. Section of bean stem through puncture area (*P*), the site of inoculation of tumor-inducing bacteria, at 5 days after treatment. Individual nuclei of proliferating tumor cells show varying histone content, associated here with mitosis and accompanying nucleohistone synthesis. At the right of the figure, transformation of normal cells to tumor cells is indicated by the divisions of polyploid nuclei of previously differentiated tissue (arrows).

FIG. 16. Section through tumorous overgrowth at 33 days after inoculation. Maturation of tumor cells is evident by the differentiation of thick-walled vascular elements (arrow) within the tumor mass. Individual nuclei show varying histone content, in this case associated with the localized accumulation of polyploid or polytene nuclei in older portions of the tumor.



(Rasch and Woodard: Basic proteins of plant nuclei)