

# Comparative Microphotometric Determinations of Deoxyribonucleic Acid in Normal and Tumorous Growth of Fern Prothalli

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General studies (7, 12-14) and cytological studies (8, 9) on fern prothallial tumors indicate that these materials present rather unique new approaches to the study of tumorous growth in plants. Both the normal and the abnormal growth forms are grown in sterile culture on synthetic media, under careful control of environmental conditions.

The abnormal growth forms isolated from gametophyte cultures of *Pteridium aquilinum* (the bracken fern) are of two general types. Growths of the first type have been regarded as merely aberrant prothallial colonies on the basis of their structural features and their potentialities for normal growth. Despite their superficial resemblance to tumorous growth, these types, designated the coralloid, pincushion, and filamentous proliferations, bear apparently functional sex organs. Also, they retain, even in prolonged culture, the ability to give rise to normal prothalli. The nuclear behavior in these forms shows no apparent deviation from that observed in normal prothalli, i.e., they remain haploid, with normal divisions.

The other general type of growth form has, by various criteria, been considered tumorous (9, 13). These growths, namely, the filamentous pseudocallus and the parenchymatous callus, also have an apparently normal nuclear condition when first isolated, showing a haploid number of chromosomes in normal mitosis. However, after the third or fourth subculture, the nuclear condition changes in these tumorous growths. A process of endoreduplication leads to an increase in chromosome number that becomes evident in all the dividing cells. The ultimate condition reached is a variable aneuploidy between the triploid and tetraploid

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levels. A similar pattern of events occurs also in comparable tumorous growths derived from prothallial cultures of *Osmunda cinnamomea* (the cinnamon fern).

When first isolated, the filamentous form of the *Pteridium* tumor produces normal-appearing prothallial regeneration, but concomitant with the nuclear change this regenerating ability is lost.<sup>1</sup> Thus, there was an apparent correlation between nuclear normalcy and the ability to achieve normal morphogenetic expression. Further support for this correlation came out of cytological examination of very rarely occurring normal-appearing prothallial regeneration on an old strain of tumor after the nuclear change. The nuclear figures in this morphologically normal regeneration were again quite normal, and the chromosome number was very nearly if not exactly euploid, though at the diploid level. Since the divisions in the tumor were apparently all of the higher aneuploid level and were quite abnormal in appearance, there was no evidence as to the origin of the diploid cells in the regeneration. Nevertheless, the occurrence of normalcy in morphogenetic expression was again correlative with apparent normalcy of nuclear behavior.

On the basis of these previous observations it was felt that a more thorough survey of the nuclear types comprising both the normal and abnormal growths would permit the reaching of some conclusions about the role of such gross nuclear changes in relation to the observed growth forms.

## MATERIALS AND METHODS

*Plant materials.*—The gametophytes of *Pteridium aquilinum* var. *latiusculum* (Desv.) Underw. and *Osmunda cinnamomea* L. were grown in sterile culture from spores collected in the field. The tumorous and other abnormal growths, earlier isolated from gametophyte cultures (7, 13), were from the stock lines of these various cultures being carried at the Harvard Biological Laboratories.

<sup>1</sup>The parenchymatous form, by definition, cannot regenerate directly, since the filamentous condition is a forerunner of the thalloid; however, the parenchymatous and filamentous forms interchange readily in culture.

*Cytological and photometric technics.*—All the materials were fixed in Carnoy (3 parts absolute alcohol:1 glacial acetic acid) for 1 hour, washed several times in 95 per cent alcohol, and then run through 100 per cent alcohol into xylol and embedded in paraffin. All specimens were sectioned at  $16 \mu$ , so as to accommodate adequately the largest of the nuclei in sufficient numbers.

The preparation of the Feulgen reagent and the staining were carried out essentially as described by Stowell (15). Twelve minutes of hydrolysis at  $60^{\circ} \text{C}$ . in 1 N HCl was found to be in the optimal range for all the materials. All slides were stained for 1 hour.

Relative amounts of deoxyribonucleic acid (DNA) in individual nuclei were measured by the apparatus shown by Pollister (10, Fig. 2). The photometric measurements were made through a central plug of the nucleus, the plug diameter being as nearly 0.60 of the mean nuclear diameter as possible, as suggested by Alfert (1). The nuclei measured were, in general, very nearly spherical.

The sections were mounted in an oil having a refractive index of 1.572, which was an exact match for the nuclei. However, in this medium the chloroplasts were mismatched. Since generally they were irregularly distributed in the region of a nucleus, they would have constituted a variable source of error due to loss of light through scattering, had the measurements been made in the conventional way. This difficulty was avoided in the following way: The first measurement ( $I$ ) was made through a plug of the nucleus, using the  $546\text{-m}\mu$  mercury line. Then, without moving the specimen, a second measurement ( $I_0$ ) was made through exactly the same plug at another wave length, which in this case was the  $643.8\text{-m}\mu$  cadmium line, where the absorption of the Feulgen stain is very low.<sup>3</sup> The light source was an FH-4 mercury-cadmium lamp. The two lines were separated by liquid filters, such as described by Pollister and Ornstein (11). In balancing the intensities of the two lines with respect to phototube response (by varying the densities of appropriate filters) the  $643.8\text{-m}\mu$  line was made approximately two-thirds the intensity of the  $546\text{-m}\mu$  line. This permitted the use of larger scale deflections for both  $I$  and  $I_0$ . These two measurements would have sufficed for any one determination had the ratio of the intensities of the two lines been known and fixed. In practice, however, there was found to be some instability between the two lines. Consequently, the ratio was checked immediately after each pair of nuclear readings by turning the slide to a clear area, away from the tissue, and taking another two readings. The  $I_0$  was then adjusted according to this ratio.

The subsequent calculations, then, were made according to the formula of Swift (16), the amount of DNA per nucleus in arbitrary units being equal to  $EC^2/F$ ,  $E$  being the extinction,  $C$  the radius of the measured plug, and  $F$  the fraction of the total nuclear volume represented by the plug.

As a control, all slides carried serial sections of the same prothallus, which was of the same species as the experimental material being studied. The presence of cytoplasmic inclusions greatly facilitated the selection of intact, uncut nuclei. Special care was taken to assure uniformity of conditions throughout. There were no appreciable differences between the controls from the several separate slides within a species. Consequently, the data were considered equivalent for the purposes of the present investigation and are presented with no further adjustment or alteration.

<sup>3</sup>Theoretically, any other wavelength could be used for the purpose if it were used consistently for all measurements which are to be compared and if it were sufficiently removed from the first wavelength to have an appreciably different position on the absorption curve.

## RESULTS AND DISCUSSION

The results of the measurements of DNA in individual nuclei of the prothallus of *Pteridium* and the various abnormal growths isolated from prothallial cultures of *Pteridium* are shown in Chart 1.

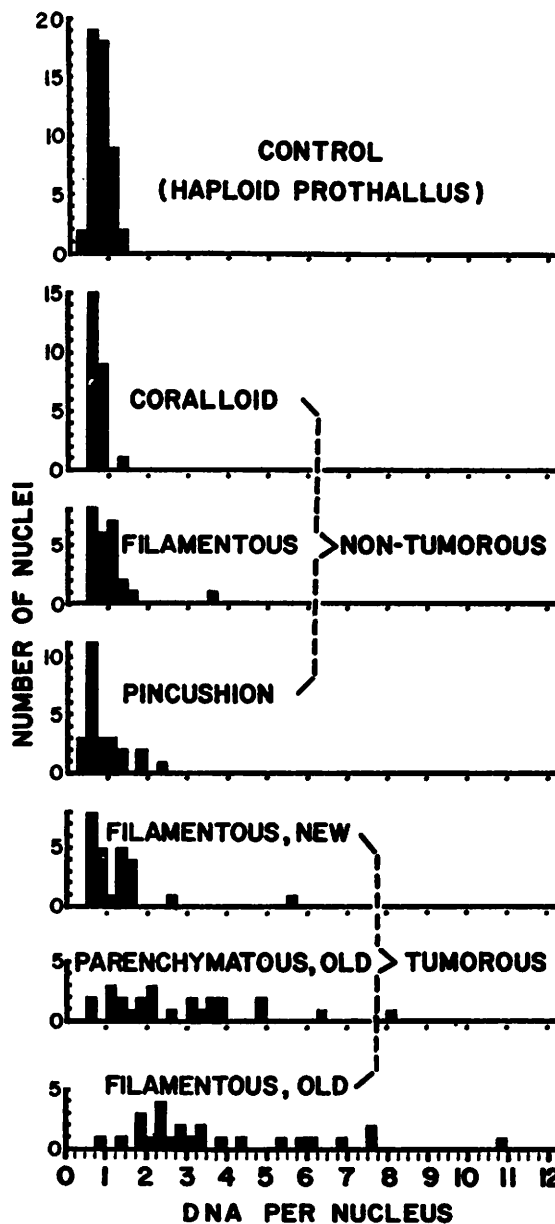


CHART 1.—Relative amounts of DNA in individual nuclei of various growth forms of *Pteridium aquilinum*. The amounts of DNA per nucleus in arbitrary units are shown on the abscissa, and the numbers of nuclei in the various classes on the ordinate. The spread of DNA values in the normal prothallus (top graph) is compared with that in three aberrant prothallial forms (next three graphs) and in three tumorous forms (bottom three graphs). The "new" tumor was in its first passage in culture, whereas the "old" tumors had undergone several passages.

A total of 50 nuclei were measured in the normal prothallus controls, which are grouped. The control serves to define the approximate limits of the range of DNA values found in the normal prothallus, the one peak probably being near the 1 C level in this haploid tissue. The lack of a second peak would suggest that in this material there is little or no accumulation of nuclei at the 2 C level, i.e., that division probably occurred quite soon after synthesis of DNA was completed or that DNA synthesis takes place rapidly near the end of interphase. However, this spread of values may be used as a reference for the other materials.

In the nontumorous abnormal growths, the coralloid, filamentous, and pincushion proliferations, the general distribution and spread of DNA values can be seen to be essentially the same as those in the controls, with some slight variation. The possible deviations from the normal conditions are, however, at a level which is difficult to interpret biologically.

Chromosome counting in these growth forms has quite consistently revealed the haploid number. Earlier observations (9) had shown, however, the occasional occurrence of endoreduplication of normal prothalli, as seen in auxin-induced divisions. It is possible, therefore, that in these nontumorous forms there may be some increased tendency toward endoreduplication, which would, of course, be undetected in chromosome counting unless those cells were to divide again. This would explain the possible slight tendency toward higher values. However, the major range of values in these nontumorous growths does coincide quite well with that found in the normal prothallus. This would indicate, therefore, that these tissues do remain predominantly haploid.

In rather striking contrast to this normal pattern are the ranges of DNA values in the old strains of the tumorous types, i.e., the filamentous pseudocallus and the parenchymatous callus. These are the forms in which the chromosome numbers had increased to a variable aneuploidy in the 3 n to 4 n range. Many of the DNA values in these tumors fall into the approximate 3 C to 4 C range. Since these measurements were all made on interphase nuclei, during which time synthesis of DNA is presumably occurring,<sup>3</sup> one might expect to find a spread of values which represent intermediates in synthesis, as well as a range of basic values due to the observed aneuploidy. There is no way of separating one type of variability from the other, since under any conditions it would be impossible to rule out the occurrence of synthesis. Also, of course, there is always superimposed upon any such biological variability an additional source

of variability due purely to instrumental and technical error. However, the patterns of distribution of DNA values in the tumors are still quite characteristic and meaningful at the level at which they are being interpreted here. While a large number of values are in the 3 C to 4 C range, many higher values are also present. These may represent synthesis of DNA, probably in large part preceding division, although the possible existence of higher endopolyploid levels cannot be excluded.

An interesting departure from that which could be predicted on the basis of earlier chromosome counts was the occurrence of an appreciable number of nuclei which had amounts of DNA in the normal haploid range, in the old strains of tumors. Once the nuclear change had occurred in the tumor strains that were studied cytologically, apparently all dividing cells were of the same new type with high chromosome numbers. The subsequent occurrence of cytologically normal, though diploid, regeneration was therefore not conclusively explained at the time. However, the DNA measurements indicate a rather wide range of nuclear types within a tumor. Indeed, they show a type of heterogeneity of cells which will be of extreme importance in the interpretation of observations on this type of tumorous tissue.

The existence of a full spectrum of nuclear DNA levels, from the 1 C to 2 C range on up to 8 C and above, points out at least one aspect in which a cell population such as comprises these relatively homogeneous-appearing tumor masses may be extremely heterogeneous. It seems reasonable to assume that similar heterogeneity may exist in other aspects also. In any case, any interpretation of a varied response locally within such a cell population, as for example the apparent "recovery" from the tumorous condition, must take this heterogeneity into consideration (e.g., see White, in "Discussion," p. 125 of [3]). Consequently, the selection of existent cell types becomes a highly probable explanation of such visible changes. Other implications of this will be discussed later, when the *Osmunda* tumors are considered.

The basic differences in the DNA patterns of

<sup>3</sup> Since telophases are extremely difficult to find and identify as such in serially sectioned prothalli, there are no measurements on telophases as compared with prophase in the *Pteridium* data presented here. However, in *Osmunda*, where the DNA concentration is higher, it was possible to measure nuclei in prothallial whole mounts. Within limits of experimental error, the DNA amounts in prophase were twice that of telophases, indicating that synthesis of DNA does occur during interphase, and that the values obtained in the normal prothallial controls therefore do represent a true 1 C to 2 C spread.

the normal prothallial and the old strain tumorous growths are quite obvious and characteristic. The question of the etiological relationship of polyploidy to tumorization has been raised by many, and various answers have been suggested. These have ranged from the *sine qua non* condition of Winge (17), which has, however, been shown in various observations not to be an invariable attribute of tumorous growth (e.g., [2]), to the other extreme in Klein's (see "Discussion," p. 78 of [5]) extension of Coleman's (4) observations. Coleman, in a study of the nuclear cytology of normal stem tissue, pointed out the existence of endopolyploidy in the tissues from which crown gall might originate. Klein then suggested that this was the source of the polyploidy in the crown gall tumors which he studied, i.e., that it was carried over by division of polyploids which were pre-existent in the host tissue. There is, however, only circumstantial evidence bearing upon the case, and the relationship must remain vague owing to the nature of the materials studied.

In the fern prothallial tumors, however, a more fortunate situation prevails, and the relationship of the polyploidy is more readily demonstrated. As was shown in Chart 1, in the DNA values of the normal prothallus, no levels as high as those found in the tumors exist in the tissue of origin. Also, extensive surveys of nuclei in whole mounts of normal prothalli failed to show any high DNA values (unpublished data). Thus, the polyploidy clearly cannot be carried over. Further, a DNA study of a newly isolated tumor in its first passage in culture shows that at the outset the new tumor is essentially in the normal haploid range, as shown in Chart 1. The histogram of this new tumor also shows the beginnings of the accumulation of a second class of nuclei by the appearance of a prominent second peak which is not evident in the normal prothallus. This would suggest that nuclear division is not occurring immediately upon reaching the 2C level, that an uncoupling of the separate processes involved in cell multiplication is beginning to occur. This study, then, constitutes more positive evidence for the earlier contention that, in this case, the occurrence of polyploidy is not associated with the initiation of the tumorous habit, either actively or passively, and that it is a secondarily acquired condition (9).

The results of similar DNA studies on normal and tumorous growth of the prothalli of *Osmunda cinnamomea* are shown in Chart 2. The normal gametophyte control shows a bimodal distribution, i.e., a prominent 1C peak and a definite 2C peak, with some indication of values higher than 2C. This was the species in which the more fre-

quent occurrence of endoreduplication was observed earlier (9). The DNA data would seem to confirm this.

The range of DNA values in the "old" tumor in *Osmunda* presents essentially the same pattern as has already been seen in the Pteridium tumors. The frequency of occurrence of certain values is, however, somewhat different. This could be due to any of the following: a greater number of intermediates in synthesis in this extremely rapidly growing tissue, a slower rate of synthesis, or a sampling error of the type that will be discussed later. Nevertheless, the pattern is characteristic, showing a wide range from the normal to a considerable spread of high values.

The other tumor strain, designated here as the "oldest," was the original strain isolated by Morel and Wetmore (7) 7 years earlier, and which was

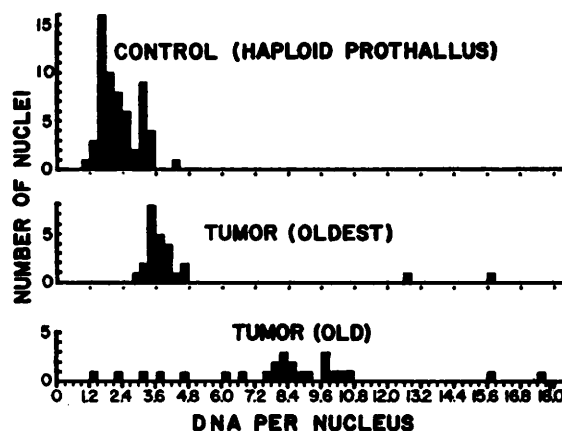


CHART 2.—Relative amounts of DNA in normal and tumorous growth of prothalli of *Osmunda cinnamomea*, plotted as in Chart 1.

later observed to be a high, variable aneuploid (9) like the Pteridium tumors. Now, obviously, the tissue is quite homogeneous and of a lower chromosome number. Thus, the strain has, in long continued culture, changed its nuclear characteristics again. On the basis of the correlations between the observed chromosome numbers and the DNA values in the Pteridium material, it may be assumed that this strain was at one time like the other "old" tumors with respect to DNA values. The question then arises how the selection of the present nuclear type occurred, since it certainly must have been a selection from the wide range of types existent in the tumor. Conceivably, many types of selection are possible, as, for example, some favorable gene combination having been reached through random shuffling of chromosomes, which combination then outgrew the rest of the population.

Perhaps a somewhat more obvious explanation is a purely passive or accidental type of selection. The data show a range of DNA levels; it should further be pointed out that these various classes do not always occur intermixed in a completely random distribution. Instead, DNA values of a certain class tend to occur together in groups, as might be expected. The differences between different areas are often quite evident even without photometric measurements, and the phenomenon presents a sectorial appearance, with sectors of varying sizes. In the process of subculturing a strain, a single fully grown culture is cut or separated into small inocula, and the strain is continued from these. Externally, one of these pieces is usually indistinguishable from the others. Yet, from the DNA studies it becomes evident that one of these sectors can, purely by chance, comprise either an entire inoculum or a large part of one. If in the next transfer this culture happens to be the one selected to continue the strain, the nuclear characteristics of the strain may be changed completely owing to this type of unconscious selection in the process of subculturing.

Similarly, this nonrandom distribution of DNA classes causes a sampling error in the attempt to determine the frequency of occurrence of the various classes within a single tumor. Clearly, a few sections may not at all be representative of the whole. Therefore, the present results are intended primarily to demonstrate simply the range or degree of diversity of nuclear types.

As to the significance of polyploidy in tumorous growth, perhaps its occurrence in normal differentiation should first be better understood. That it occurs in both forms of growth may, however, be of importance in reaching an understanding about its occurrence in either case. The same basic process, or alteration of processes, produces the condition in both cases, i.e., an uncoupling of factors involved in normal meristematic type of cell division. The differences between the two are that in normal growth the polyploidy usually escapes detection, since such cells ordinarily do not divide again, and also that such changes do not occur normally in meristematic regions, whereas in the tumorous growth the changes do occur in those cells which act in a meristematic capacity (in a limited sense) and which therefore continue to divide.

In the process of differentiation, the plant cell certainly exists in a changing physical and chemical environment. Under such conditions various organelles, as for example the nucleus, may reflect these changes. Polyploidy could be one such manifestation. To what extent are such changes limiting

in the further potentialities of the cells? Certainly polyploidy alone, at least at the lower levels of ploidy, need not be a restraint, as shown, for example, in studies on polyploid regeneration from cut shoots (6). This indicates that many cells, even though polyploid, may once again express totipotentiality if given a release from physical and perhaps other restraints. Similar conclusions can be drawn from the occurrence of apparently normal, though diploid, regeneration from these fern tumors. The additional occurrence of aneuploidy, however, may present other problems (9). What this would suggest, then, is that purely from a nuclear point of view certain of the variety of cells comprising the total entity which we recognize as a tumor might still be expected to be capable of normal expression, given the proper conditions.

#### SUMMARY

DNA in individual nuclei of normal and tumorous growth of fern prothalli *in vitro* has been determined by photometric measurements of the Feulgen reaction. The normal prothalli show DNA amounts in the 1 C to 2 C range, as do certain nontumorous aberrant growth forms. Newly isolated tumor strains are essentially like the normal prothalli in DNA pattern, but in subsequent passages in culture they develop a spectrum of DNA values from the normal to much higher levels. This shows that polyploidy is neither causal to, nor even present in, the initiation of this type of tumorous growth, but that it is clearly a secondarily acquired and perhaps diagnostic characteristic. The persistence of some cells in the normal range and the heterogeneity of the cell population comprising the tumor are considered significant factors in the interpretation of varied behavior and apparent "recovery" from the tumorous condition.

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