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A Cytochemical Study of Nucleic Acids in Plant Cells
VII. Causal Analysis of Negative Feulgen Staining

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Feulgen staining method (FEULGEN and ROSSENBECK, 1924) has been widely used for detection of DNA in histochemical preparations by many cytologists, because the method is the most specific for DNA among several methods, though the mechanism of this staining is still not completely cleared.

It has been reported by several authors that the color intensity of Feulgen staining in histochemical preparations is, however, affected by many factors such as periods and temperature of acid hydrolysis, co-existing proteins and some inhibiting substances and others (HILLARY, 1939; MILOVIDOV, 1949). As in the case of histochemical preparations, the intensity of Feulgen reaction color developed *in vitro* is also affected by many factors (CASPERSSON, 1932; SIBATANI, 1950; SHINKE, ISHIDA and UEDA, 1957; ISHIDA, 1959).

On the other hand, it has been reported by several authors that cells of certain plants are not stained with Feulgen method (SHINKE and SHIGENAGA, 1933; HILLARY, 1939; MILOVIDOV, 1949). SHINKE, ISHIDA and UEDA (1957) have also observed that cells of certain algae, belonging to Conjugatae and Cyanophyta, are not stained with Feulgen method. As in the case of plant cells, similar phenomenon has also been observed in cell nuclei during a certain stage of egg development of sea-urchins and starfishes (MARSHAK, 1954, 1958).

Causal analyses on negative Feulgen staining, observed by several authors stated above, have been carried out by only a few investigators such as HILLARY (1939) in histochemical preparations and model experiments, but the results obtained by these investigators are still fragmentary. The purpose of the present study is to obtain findings on the negative Feulgen staining, using some plants by biochemical procedure.

Material and Method

As materials and methods used in this study are different according to the purpose of experiment, they will be given in appropriate places in the following pages.

Experiments and Results

1) *Effect of acid hydrolysis on nucleic acids in cells.*

It was pointed out by HILLARY (1939) and MILOVIDOV (1949) that intensity of Feulgen color of cell nuclei decreased by prolonged hydrolysis in HCl solution. This fact may be assumed as due to the extrusion of a certain amount of DNA from cell nuclei into the hydrolysing acid solution. To see whether the above assumption was correct or not, amounts of nucleic acids extruded from cells into HCl solution as well as these acids remaining in cells after hydrolysis were determined quantitatively.

Fresh materials of *Oscillatoria princeps* and *Spirogyra* sp., which were Feulgen negative algae, and *Cladophora* sp., a Feulgen positive alga, were fixed with CARNOY's fluid for 1 hour at room temperature. After fixation, materials were repeatedly washed with absolute ethanol, and ice-cold distilled water successively. Each aliquot of materials was then hydrolysed with N-HCl at 60°C for 5, 10, 15, 20, 25 and 30 minutes in each test tube. After hydrolysis, tissue residues were separated from HCl solution by centrifugation. HCl solutions thus obtained were taken to determine the nucleic acids extracted from cell into the solution.

On the other hand, nucleic acids were then extracted from tissue residues, obtained by centrifugation stated above, by heating the residues with 0.5 N-perchloric acid (PCA) for 20 minutes at 70°C (OGUR and ROSEN, 1950 ; ISHIDA, 1960, 1961a). The amount of total nucleic acids, DNA and RNA, extracted from cells into HCl solution was determined by UV-absorption, DISCHE's and orcinol methods (MEJBAUM, 1939). Details of first two methods were given in the previous paper (ISHIDA, 1954, 1959, 1960). Determination of nucleic acids remained in cell residues was also carried out by above methods.

Figure 1 shows UV-absorption curves of mixtures of both nucleic acids extracted from cells into HCl and PCA solutions. It is seen in this figure that there are no significant differences among UV-absorption curves of nucleic acids extracted from cells of *Oscillatoria, Spirogyra* and *Cladophora* into HCl and PCA solutions. These nucleic acids in both the solutions give the typical absorption curves as in the case of purified nucleic acids. Results of determination of the amount of DNA in both HCl and PCA solutions stated above with DISCHE's method are shown in Fig. 2.

In this figure, it is seen that DNA in cells of *Oscillatoria* is extracted more easily into HCl solution during hydrolysis than DNA in *Spirogyra* and *Cladophora* cells.

Figure 3 shows the amounts of total nucleic acids and RNA extruded from cells into HCl solution, and those remaining in tissue residues after hydrolysis.

It is seen in this figure that these acids are more easily extracted from *Oscillatoria* cells than the cells of other algae. From the above experiment, it is found that the stability of DNA in cell nuclei to the HCl hydrolysis is different as the kind of algae used differed. Extraction of a large amount of DNA from

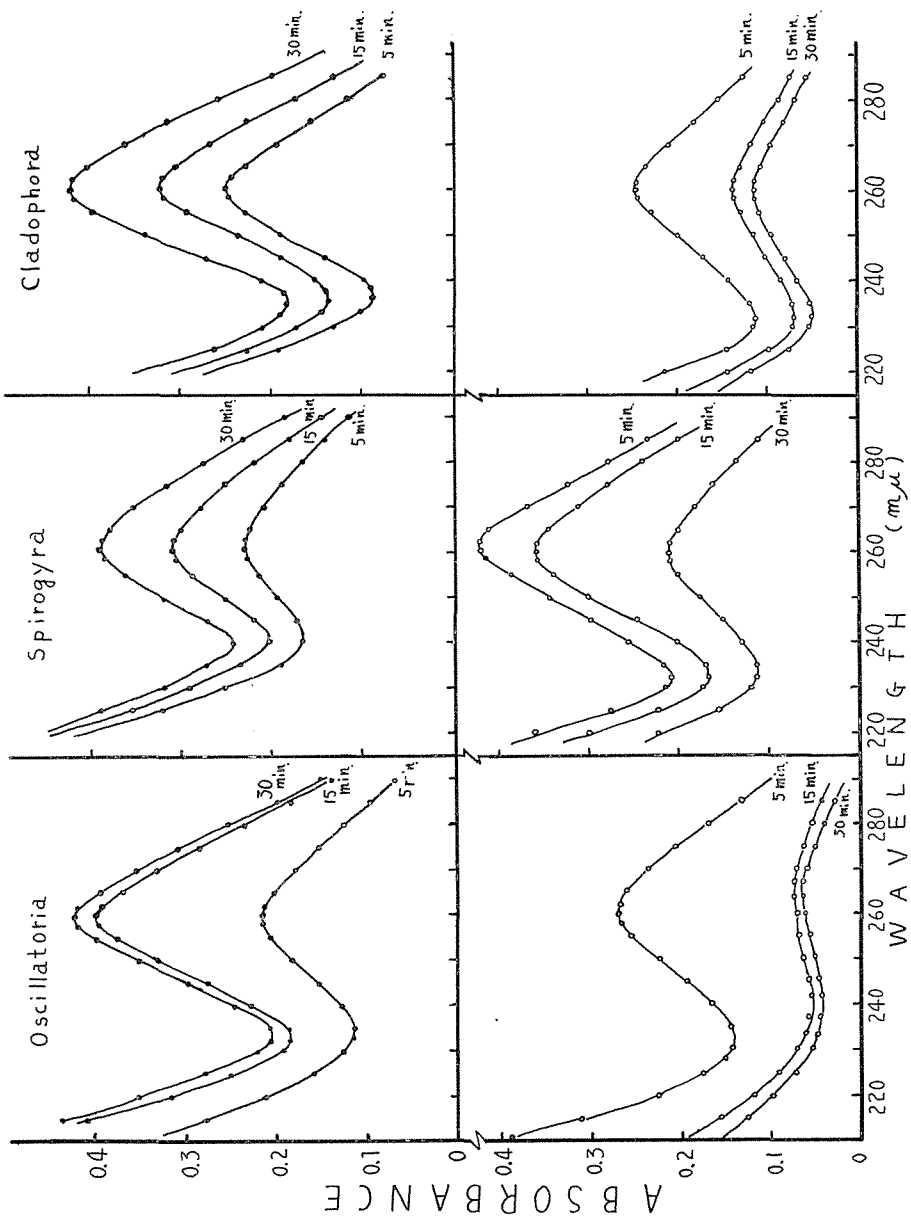

Fig. 1. Showing UV-absorption curves of nucleic acids in HCl (—·—) and PCA (—∘—) solutions. Time shown in this figure represents the period of hydrolysis with HCl.

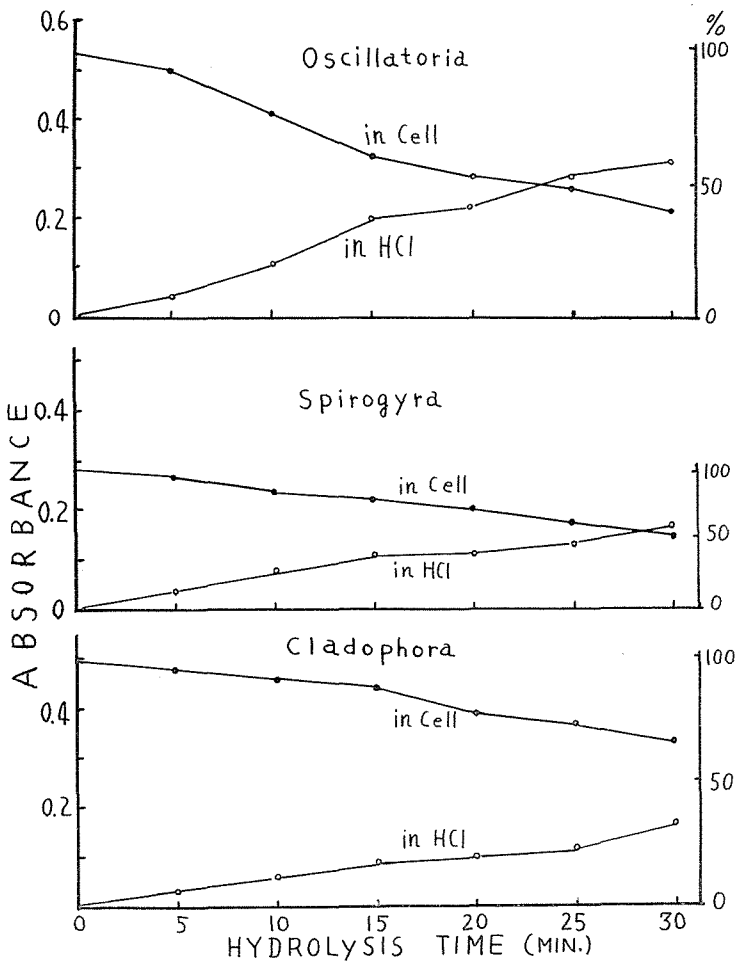

Fig. 2. Showing amounts of DNA extruded from cells into HCl and remained in cell nuclei in various periods of hydrolysis.

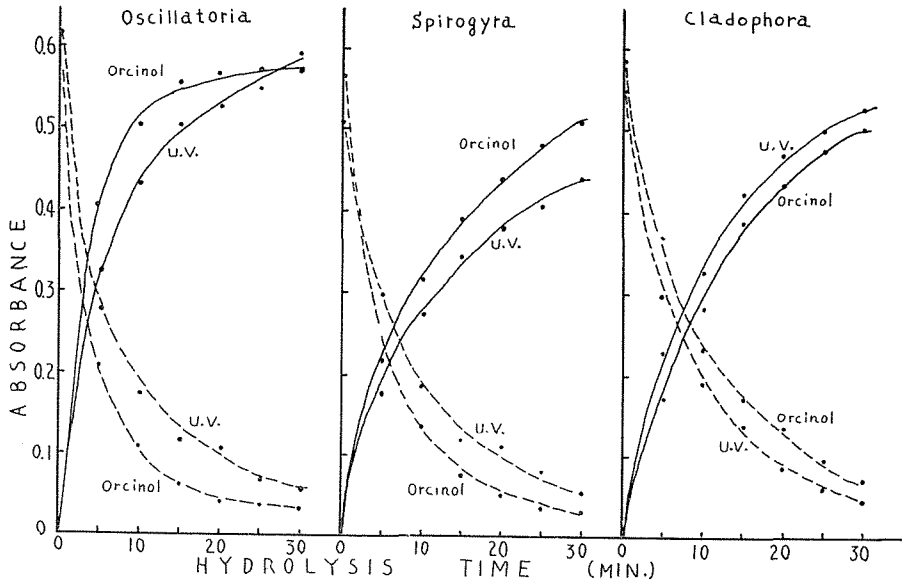

Fig. 3. Showing amouts of total nucleic acids (UV) and RNA (orcinol) extruded from cells into HCl and remained in cells in various periods of hydrolysis.

cell nuclei during hydrolysis, must be regarded as one of the causes of the loss of Feulgen stainability in cell nuclei.

2) *Effect of amino acids on the development of Feulgen color* in vitro.

It was reported by SHINKE, ISHIDA and UEDA (1957) that L-tryptophane inhibited the development of Feulgen color *in vitro*. In the present experiment, effects of various amino acids on the development of Feulgen color *in vitro* were studied.

Ten mg of DNA were hydrolysed with 20 ml of N-HCl at 90°C for 15 minutes. After hydrolysis, hydrolysed DNA solution was neutralysed by an addition of suitable amount of N-KOH, and made up to 50 ml by an addition of distilled water. Two ml of 0.1 M-amino acid solution and 14 ml of citrate buffer solution, adjusted at pH 3.0, were added to 2 ml of hydrolysed DNA solution stated above. This mixture was cooled at 10°C, and 2 ml of SCHIFF's reagent were added to this mixture. Reddish purple color developed gradually. After 1 hour, intensity of the developed color was determined at 565 mμ by a BECKMAN's spectrophotometer.

Result obtained is shown in Table 1.

It is seen in this table that these amino acids, except tryptophane, do not markedly affect the development of Feulgen color, but tryptophane inhibits the color development. Mechanism of this inhibition will be discussed later.

Table 1. Effect of amino acids on Feulgen color development *in vitro*.

Amino acid added	%	Amino acid added	%
DL-Alanine	100.0	L-Phenylalanine	96.9
L-Arginine·HCl	100.0	L-Proline	95.9
L-Aspartic acid	98.5	Hydroxy-L-proline	98.6
L-Cystine	97.9	DL-Threonine	97.8
L-Glutamic acid	99.5	L-Tyrosine	99.9
Glycine	100.1	L-Tryptophane	83.1
L-Histidine·HCl·H_2O	100.0	DL-Serine	99.3
L-Leucine	99.9	DL-Valine	97.3
L-Lysine·HCl	99.8		
DL-Methionine	98.5	DNA alone (control)	100.0

3) *Effects of proteins from some plant cells on the development of Feulgen color* in vitro.

Results obtained in the previous study show that proteins such as albumin, gelatin, casein, histone and protamine increased the intensity of Feulgen color *in vitro* (ISHIDA, 1959), though it has been reported by SWIFT (1955) that some globulin fraction from certain cell nuclei inhibit the Feulgen color development. Whether proteins isolated from cells of Feulgen negative plants inhibit the Feulgen reaction *in vitro* or not; effects of proteins from *Oscillatoria princeps* and *Spirogyra* sp. on the Feulgen reaction *in vitro* were studied. To compare the effect of proteins from Feulgen negative plants to that of proteins from Feulgen positive plants, proteins isolated from the cells of 5 mm segments of root tips of *Vicia* seedlings were used.

These proteins were isolated by SEVAG's procedure (SEVAG, 1934) from 'DNA fraction' obtained from these plant materials by a modified SCHMIDT and THANNHAUSER's method (SUGIYAMA, SHINKE and ISHIDA, 1954; SHINKE, ISHIDA and UEDA, 1957). Samples of these proteins contained some water soluble proteins and they were isolated from the samples by elution with warm distilled water, and then dried.

Two ml of 0.5% protein solution obtained by above procedures were added to 2 ml of 0.05% calf thymus DNA solution. Then 4 ml of 0.2 N-HCl were added to the DNA-protein mixture, and it was hydrolysed at 90°C for 15 minutes. After hydrolysis, a suitable amount of glycine-NaCl solution was added to the hydrolysed mixture for adjusting the pH at 2.28. Two ml of SCHIFF's reagent were added to the mixture. After 1 hour, intensity of the Feulgen color was determined.

Results obtained are shown in Table 2.

In Table 2, it is shown that proteins isolated from cells of *Oscillatoria* and *Spirogyra*, Feulgen negative algae, markedly inhibit the development of Feulgen color, while such inhibition by proteins from the root tips of *Vicia*, a Feulgen

Table 2. Effect of proteins from Feulgen negative and positive plants on the development of Feulgen color with calf thymus DNA *in vitro*.

Protein added	Absorbance	Inhibition
Oscillatoria princeps	0.402	25.8 %
Spirogyra sp.	0.431	20.5
Vicia faba (root tip)	0.502	7.4
Calf thymus DNA only (control)	0.542	100.0

positive plant, was much less than that caused by the presence of proteins from the two algae stated above. Results of paperchromatographic analysis show that at least 14 amino acids, including tryptophane, are found in proteins from both *Oscillatoria* and *Spirogyra* cells.

4) *Effects of tannin on the development of Feulgen color* in vitro.

It was pointed out by MILOVIDOV (1936) that the Feulgen stainability of cell nuclei was affected by presence of tannin. Analysis of such a phenomenon had not, however, been carried out quantitatively. In the present experiment, too, effects of tannin on Feulgen color development were studied *in vitro*.

Hydrolyzation of DNA followed by neutralyzation was carried out as in the case of Experiment 2. One ml of aqueous solution of tannin[1] in various concentrations was added to 1 ml of hydrolysed DNA solution. Seven ml of citrate buffer solution at pH 3.0 and 1 ml of SCHIFF's reagent were added successively to above DNA-tannin mixture cooled at 10°C. After 1 hour, intensity of the color developed was determined.

Result obtained is shown in Fig. 4.

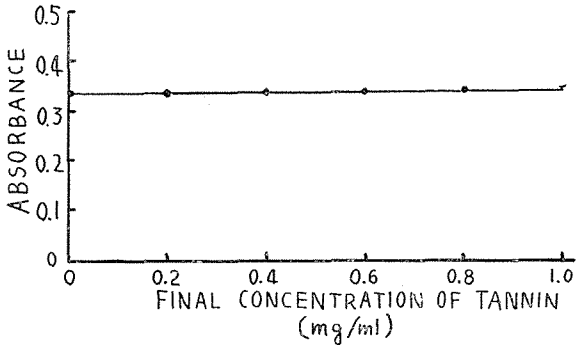

Fig. 4. Showing effect of tannin in various concentrations on the intensity of Feulgen color *in vitro*.

1) Obtained from Merck. This preparation was completely soluble in water.

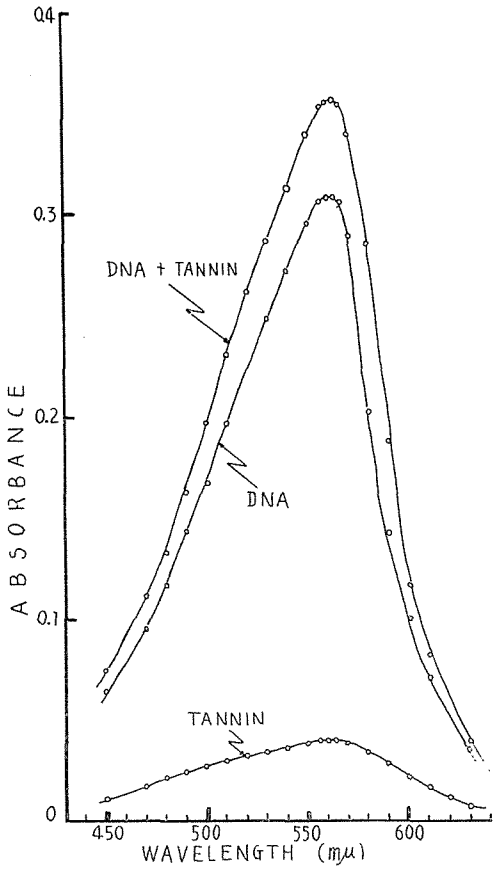

Fig. 5. Showing absorption curves of Feulgen color developed with DNA, DNA-tannin mixture and tannin only.

As seen in this figure, intensity of the Feulgen color was not affected by presence of any concentrations of tannin *in vitro*. Similar result was obtained by HILLARY (1939) who showed that tannin did not normally inhibit the staining of DNA-agar blocks after treatment with certain fixative in model experiment.

In Fig. 5, it is seen that the maximum wavelength of absorption curve of the Feulgen color developed with DNA-tannin mixture is the same with that of DNA only. Tannin itself gives the positive test with Feulgen method *in vitro*, and moreover, the maximum wavelength of its color developed locates at the same wavelength, at 565 mμ, with that of DNA as seen in this figure. It must, therefore, be mentioned that presence of tannin gives a 'pseudo-reaction' *in vitro*.

5) *Feulgen value and DNA composition.*

FEULGEN and ROSSENBECK (1924) stated that purine-sugar bonds of DNA were split as a result of the HCl hydrolysis and aldehydic substances such as ω-hydroxylevulinic aldehyde (STACEY *et al.*, 1946; OVEREND, 1950) or apurinic acid (TAMM *et al.*, 1955) were formed. These substances reacted with SCHIFF's reagent to give a colored compound. Feulgen value of DNA, therefore, correlated with the purine content of its nucleic acid (LALAND *et al.*, 1952).

In the present study, to see whether such relationship between Feulgen value and purine content can be applied to *Oscillatoria* DNA, which was low purine content, or not; Feulgen values of *Oscillatoria* DNA and calf thymus DNA, in which ratios of purines to pyrimidines of both DNAs were 0.87 (ISHIDA, 1961b) and 1.00 (CHARGAFF *et al.*, 1949) respectively, were detemined. Dische and Feulgen values of *Oscillatoria* DNA were also compared with those of calf thymus DNA.

Each constituent of DNA such as sugar, base and phosphorus was estimated independently by DISCHE's, FEULGEN's, UV-absorption and PVM-methods (SUGIYAMA, SHINKE and ISHIDA, 1954). Details of the first three methods were described in the previous papers (ISHIDA, 1954, 1956, 1959, 1960, 1961a).

Results obtained are shown in Table 3. In this table, each estimation value of DNA constituents obtained by the procedures mentioned above is represented

Table 3. Absorbance of DNA from *Oscillatoria princeps* and calf thymus per γ of DNA-P estimated by PVM method.

Estimation methods	DNA sources		Ratio of absorbance $\left(\frac{Oscillatoria}{\text{thymus}}\right)$
	Oscillatoria princeps	Calf thymus	
Dische	0.066	0.069	0.957
U. V.	0.341	0.351	0.972
Feulgen	0.075	0.095	0.789

in 'absorbance' per γ of DNA-P. As seen in this table, the value obtained by DISCHE's method in *Oscillatoria* DNA is nearly equal to that in calf thymus DNA. Similar results are shown in UV-values of both DNAs. Feulgen value of *Oscillatoria* DNA is, however, much lower than that of calf thymus DNA, that is, the ratio of both Feulgen values is 0.789 as seen in Table 3. This value is in comparative accordance with that obtained in the ratio of purines to pyrimidines of *Oscillatoria* DNA (ISHIDA, 1961b). As above mentioned, the ratios of purines to pyrimidines of *Oscillatoria princeps* DNA and calf thymus DNA are 0.87 and 1.00 respectively. These facts show that the intensity of Feulgen color depends on the purine content of DNA.

From the results of these biochemical analyses, it is assumed that intensity of the Feulgen staining of cell nuclei depends on the purine content of DNA in cell nuclei.

6) *Concentration of DNA in cell nuclei.*

It was assumed by HILLARY (1939) and MILOVIDOV (1949) that one of the causes of negative Feulgen staining of cell nuclei was partly of the low concentration of DNA in cell nuclei, that is, the intensity of Feulgen staining was reduced when staining materials were widely dispersed in the large nucleus. But this assumption expressed by these authors had no experimental basis.

In the present study, therefore, DNA concentration (amount of DNA per nucleus) in cell nuclei of some Feulgen negative plants such as *Oscillatoria limosa*, *Spirogyra* sp. and *Marchantia polymorpha* were determined, and the values obtained were compared with in *Vicia* root tip cells.

Quantitative isolation of DNA from these plant materials was carried out by a procedure of SCHMIDT and THANNHAUSER's method with a slight modification by us (SUGIYAMA, SHINKE and ISHIDA, 1954). Amount of DNA per cell isolated

from these materials was estimated by procedures of DISCHE's (ISHIDA, 1954, 1956), FEULGEN's methods (ISHIDA, 1959), UV-absorption method (ISHIDA, 1960, 1961a) and PVM-method (SUGIYAMA, SHINKE and ISHIDA, 1954).

In Table 4, the values of DNA content per cell obtained by these estimation methods are represented as mean value. In the last column of this table, concentration of DNA in each of the cell nuclei is represented.

Table 4. DNA concentration in cell nuclei of some plants.

Species	DNA content per cell ($\times 10^{-9}$ mg)	Nuclear volume (μ^3)	DNA concentration ($\times 10^{-12}$ mg/μ^3)
Oscillatoria limosa	8.1	158.3*	51.2
Spirogyra sp.	2.9	112.9	25.7
Marchantia polymorpha (Thalli)	0.48	28.6	16.8
Vicia faba (Root tips)	64.4**	405.2	158.9

* Volume of central bodies was determined.

** Determination of DNA per cell was carried out by SHINKE and ISHIDA (unpublished) with a modified SCHMIDT and THANNHAUSER's method and PVM-method for estimation of nucleic acid-P.

As seen in this table, concentration of DNA in cells of three Feulgen negative plants used in this study is of the same level in all cases, but much lower than those of *Vicia* root tips. From the results of Experiment 6, it is assumed that Feulgen color intensity partly depends on the concentration of DNA in cell nuclei.

Discussion and Conclusion

Mechanism on chemical basis of Feulgen staining has not still been cleared, but this staining method has widely been used for DNA detection in cells by many cytologists. Cell nuclei of most organisms give the positive staining with FEULGEN's method. However, it has been observed by several authors that cell nuclei of certain plants are not stained with this method (BOAS and BIECHELE, 1932; SHINKE and SHIGENAGA, 1933; HILLARY, 1939; UEDA, 1956).

It was reported in the previous paper that cells of *Oscillatoria limosa*, *Marchantia polymorpha* and *Spirogyra* sp. contained certain amount of DNA, while these cell nuclei were not stained with FEULGEN's method, and that DNA isolated from these plants gave positive test with FEULGEN's method *in vitro* (ISHIDA, 1961a). Moreover, it must be noted here that amounts of DNA contained in a single cell of *Oscillatoria limosa* and *Spirogyra* sp. were the same level with those of cells of Feulgen positive animal organs such as pancreas, thymus, spleen and kidney of rat and fowl liver (*cf.* LESLIE, 1955). These facts show, at least, that negative Feulgen staining of cell nuclei in these plants is not due to the absence of DNA, though the causes of this phenomenon have not been cleared yet.

The causes of negative Feulgen staining have been discussed by HILLARY

(1939) and MILOVIDOV (1949), but basis of these discussions is rather morphological than cytochemical. It may be stated that the causal analyses of the negative Feulgen staining, based on the cytochemical experiments, have not been carried out yet. In the cytochemical study, reported in the present paper, several factors which bear important relations to the negative Feulgen staining are found.

It was assumed by MILOVIDOV (1949) that chemical difference of DNA itself must be regarded as one of the factors which carried an important relation to the development of Feulgen color in histochemical preparations. More recently, LALAND (1952) and TAMM *et al.* (1955), from the results of their biochemical studies of DNA of several different sources, arrived at a conclusion that intensity of Feulgen reaction color correlated with the purine content of DNA. On the other hand, it was reported that ratio of purine to pyrimidine of DNA was nearly one in most organisms (CHARGAFF, 1955), while results obtained in the previous paper showed that this ratio in *Oscillatoria* DNA was much lower than one. In the present study, similar results were obtained in the ratio of Feulgen value of *Oscillatoria* DNA to that of calf thymus DNA in which the ratio of purine to pyrimidine was one.

From the experimental results stated above, it would be concluded that the negative Feulgen staining in *Oscillatoria* cells was partly due to the fact that purine content of DNA of this alga was low.

It was reported by BRINGMANN (1950) that a fairly large amount of DNA was eluded from *Oscillatoria* cells into HCl solution during hydrolysis. He also pointed out that Feulgen negative staining was due to such easy elusion of DNA during hydrolysis. Results of the present investigation show that part of DNA in cells extrudes from cells into HCl or the hydrolysing agent, and there is marked difference in the relative amount of extruded DNA in HCl between the Feulgen negative alga, *Oscillatoria*, and Feulgen positive alga, *Cladophora*. The cause of this difference was not cleared in the present experiment, however.

Results obtained in the present experiment showed that the proteins isolated from *Oscillatoria* and *Spirogyra* inhibited the development of Feulgen color more strongly than those from *Vicia* root tips. Inhibition of the development of Feulgen color by the presence of proteins was also reported by SWIFT (1950, 1955) who showed that certain globulin fraction partially inhibited the Feulgen color development and that Feulgen color intensity of certain cell nuclei was increased by a loss of proteins.

COHEN (1945) showed that hydrolysed DNA combined with tryptophane in acid solution. The result of the present experiment seemed to accord with that obtained by COHEN stated above. Most amino acids, contrary to tryptophane, did not give any significant effect on the development of Feulgen color.

It was pointed out by MILOVIDOV (1949) that one of the causes of negative Feulgen staining observed in many plant cells was due to presence of tannin. MILOVIDOV (1936) observed that the intensity of Feulgen staining in tissue sec-

tions was decreased after they were treated with tannin solution, and assumed that tannin hindered the penetration of SCHIFF's reagent.

In HILLARY's experiments, it was shown that tannin did not inhibit the Feulgen color development in cytological preparations when cells were fixed with CARNOY's fluid. Results obtained in the present experiment *in vitro* showed that tannin did not inhibit Feulgen reaction.

As an important factor on the causes of negative Feulgen staining, DNA concentration of cell nuclei must be also taken into consideration. Results obtained in the present investigation have shown that there were some significant differences in DNA concentration among cell nuclei of Feulgen negative plants used in this study. However, significant difference in DNA concentration between the cells of Feulgen positive and negative plants used was found, that is, DNA concentration in cell nuclei of *Vicia* root tips was much higher than that in *Oscillatoria limosa*, *Spirogyra* sp. and *Marchantia polymorpha*.

An assumption that low concentration of DNA was a factor of negative Feulgen staining has been expressed by several authors. SWIFT (1955) stated that DNA concentration in certain cell nuclei was obviously too diluted to produce visible staining. MARSHAK (1958) expressed a view based on an observation of echinoderm eggs that negative Feulgen staining found in this animal was not to be accounted for by dispersion of small amount of DNA in a large cell nucleus, but due to degradation of polymerized DNA in certain developmental stages of these eggs. These assumptions stated above were different from one another, but concluded from the results of the present experiments that DNA concentration of cell nuclei could be regarded as one of the important factors which determine the Feulgen stainability of cell nuclei in histochemical preparations.

It has been shown in this paper that there are several factors which reduce the Feulgen color development *in vitro*. Results of the present experiments show that intensity of the Feulgen color is correlated directly with the purine content of DNA, and certain proteins from Feulgen negative plants markedly inhibit the Feulgen color development *in vitro*. It has also been found that amount of DNA eluded from cells into HCl during hydrolysis is different as plants used differed, that is, DNA in *Oscillatoria* cells, a Feulgen negative alga, is extracted more easily from cells into HCl solution during hydrolysis than that in *Cladophora* cells, Feulgen positive alga, and that concentration of DNA in cell nuclei is an important factor which determines the Feulgen color intensity.

From the results of the present study, it is assumed that negative Feulgen staining is not caused by only one factor but caused by several factors, according to findings we have made in the present study here recorded.

Summary

1) Causal analyses of negative Feulgen staining of cell nuclei were carried

out by biochemical procedure. Several factors affecting the Feulgen color development were analysed in the present study.

2) Amounts of DNA extruded from cell nuclei into HCl solution, and those remained in cell nuclei after various periods of HCl hydrolysis were determined. Similar estimations were carried out on RNA and total nucleic acids. DNA in cells of *Oscillatoria*, a Feulgen negative alga, was extruded more easily from cell nuclei into HCl solution during hydrolysis than DNA in cells of *Cladophora* which was Feulgen positive alga.

3) Tryptophane inhibited the Feulgen color development *in vitro*, but other amino acids used were not significantly affected of the color development.

4) Proteins isolated from *Oscillatoria princeps*, *Spirogyra* sp. inhibited the Feulgen color development *in vitro* more than that from root tips of *Vicia* seedlings, a Feulgen positive plant.

5) Feulgen color development was not affected by the presence of tannin *in vitro*. Tannin itself, however, showed positive in test with Feulgen method *in vitro*.

6) Relation between purine content and Feulgen value of *Oscillatoria* DNA was studied. Result obtained showed that intensity of Feulgen color depended on the purine content of DNA.

7) Significant difference in DNA concentration of cell nuclei between Feulgen positive and negative plants used was found.

8) Results obtained in the present study showed that negative Feulgen staining of cell nuclei was not caused by only one factor but caused by several factors.

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Literature Cited

BOAS, F., & O. BIECHELE, 1932. Biochem. Z., **254**: 467.
BRINGMANN, G., 1950. Planta, **38**: 541.
CASPERSSON, T. O., 1932. Biochem. Z., **253**: 97.
CHARGAFF, E., 1955. The Nucleic Acids, I. Academic Press.
COHEN, S. S., 1945. J. Biol. Chem., **167**: 691.
DISCHE, Z., 1931. Abderh. Handb. d. biol. Arbeitm., 355.
FEULGEN, R., & H. ROSSENBECK, 1924. Hoppe-Seyl. Z., **153**: 203.
HILLARY, B. B., 1939. Bot. Gaz., **101**: 276.
ISHIDA, M. R., 1954. Mem. Coll. Sci. Univ. Kyoto, (**B**), **21**: 55.
——— 1956. Ibid., (B), **23**: 93.
——— 1959. Cytologia, **24**: 107.
——— 1960. Ibid., **25**: 481.
——— 1961a. Ibid., 28. (In press.)

ISHIDA, 1961b. Mem. Coll. Sci. Univ. Kyoto, (B), **28**: 73.
LALAND, S. G., W. G. OVEREND & M. WEBB, 1952. J. Chem. Soc., **1952**: 3224.
LESLIE, I., 1955. The Nucleic Acids, II. Academic Press.
MARSHAK, A., 1954. Biochim. Biophys. Acta, **15**: 584.
——— 1958. Symp. Soc. Exp. Biol., XII, 205.
MEJBAUM, W., 1939. Zeits. phys. Chem., **258**: 117.
MILOVIDOV, P. F., 1936. Protoplasma, **25**: 570.
——— 1949. Protoplasma-Monographien.
OGUR, M., & G. ROSEN, 1950. Arch. Biochem., **25**: 262.
OVEREND, W. G., 1950. J. Chem. Soc., **1950**: 2769.
SCHMIDT, G., & S. J. THANNHAUSER, 1945. J. Biol. Chem., **161**: 83.
SHINKE, N., M. R. ISHIDA & K. UEDA, 1957. Proc. Intern. Genetics Symp. 1956; Suppl. Vol of Cytologia, 156.
——— & M. SHIGENAGA, 1933. Cytologia, **4**: 189.
SIBATANI, A., 1950. Nature, Lond., **165**, 355.
STACEY, M., R. E. DERIAZ, E. G. TEECE & L. F. WIGGINS, 1946. Ibid., **157**: 704.
STOWELL, R. E. 1954. Stain Technol., **20**: 45.
SUGIYAMA, H., N. SHINKE & M. R. ISHIDA, 1954. The Bot. Mag. (Tokyo), **67**: 138.
SWIFT, H., 1950. Physiol. Zool., **23**: 169.
——— 1955. The Nucleic Acids, II. Academic Press.
TAMM, C., M. E. HODGES & E. CHARGAFF, 1952. J. Biol. Chem., **195**: 49.
UEDA, K., 1956. Mem. Coll. Sci. Univ. Kyoto, (B), **23**: 87.