MEMOIRS OF THE COLLEGE OF SCIENCE, UNIVERSITY OF KYOTO, SERIES B
Vol. XXI, No. 1, Article 9, 1954

A Cytochemical Study of Nucleic Acids in Plant Cells

I. A critical study of DNA determination with Dische reaction.

By

Masahiro Ruè ISHIDA

Department of Botany, University of Kyoto

(Received March 20, 1954)

Results of the biochemical analyses of cellular components carried out during last decade show that two types of nucleic acids, desoxyribonucleic acid (DNA) and pentosenucleic acid (PNA) are contained widely in animal cells. Biochemical methods of analysis, however, do not give us any knowledge of the localization of these acids in morphological elements of the cells, such as nucleus, cytoplasm, mitochondria etc. Many investigators, therefore, employed differential centrifugation, and microscopical methods, such as microspectrophotometry, to clear the distribution of the acids in cellular organelles (Caspersson, 1936; Claude and Potter, 1943; Pollister and Ris, 1947).

Considering from results of the investigations by Brachet (1950), Caspersson (1950) and others, it is highly probable to assume that nucleic acids and their derivatives play an important rôle in biological phenomena of cells, such as division, growth and differentiation.

These investigations, however, have mainly been carried out on animal cells and only rarely on plant cells. It is obvious, therefore, that further investigation is necessary to obtain a clearer knowledge on the physiological function of nucleic acids in plant cells.

In this series of investigation, it is intended to study not only nucleic acids content of plant cells but the localisation of the substances in the protoplasts by cytochemical methods and obtain some knowledge on the function of nucleic acids in plant cells (cf. Sugiyama, Shinke and Ishida, 1954).

Among biochemical methods of quantitative determination of DNA, Dische's colorimetric method (1931) has been used by several investigators, such as Masayama, Yokoyama and Shudo (1940) and Schneider (1945), but results of our preliminary experiment show that the color developed as a result of Dische reaction is not stable. In this paper, therefore, it is intended to find a better procedure than that of Dische by employing his reagent.

Experimentation

The reagent used in the present experiments was prepared as mentioned by Dische (1931), e. g. 1 volume of conc. H_2SO_4 was added to 39 volumes of 2% diphenylamine solution in glacial acetic acid. To determine the amount of DNA, 2 volumes of the reagent were added to 1 volume of sample solution containing Na-DNA, then the mixture was heated in water bath. After heating, the mixture which became blue in color, was cooled in ice water. Then, the absorbance of the color was determined with a Beckman spectrophotometer.

Experiment 1. Effect of the time of heating.

Na-DNA was dissolved in N/10-KOH solution (0.3 mg/ml). The Dische reagent was added to this and was heated for 10, 20, 30 and 40 minutes at 90°C. The absorption curves of the developed color in these four cases are shown in Fig. 1.

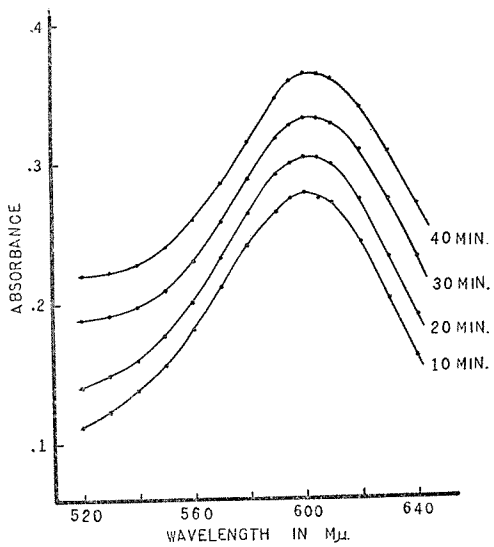

Fig. 1. Absorption curves of the color developed with Dische reaction. The time of heating is noted in each curve.

As shown in Fig. 1., the maxima of the absorption curves are at 600mμ in these cases. It must be mentioned here that the mixtures heated for 10 and 20 minutes are clear and blue while those heated for 30 and 40 minutes are dark and reddish-blue which is hardly regarded as typical reaction color. (cf. Dische, 1931; Masayama et al, 1940). It is recommended, therefore, not to heat the mixtures longer than 20 minutes.

It is highly probable, on the other hand, to consider that the development of the color is not complete when the mixture is heated only for a short time. It is necessary, therefore, to clear the relation between the time of heating and the

development of the color. The results of the experiment on this problem are presented in Fig. 2.

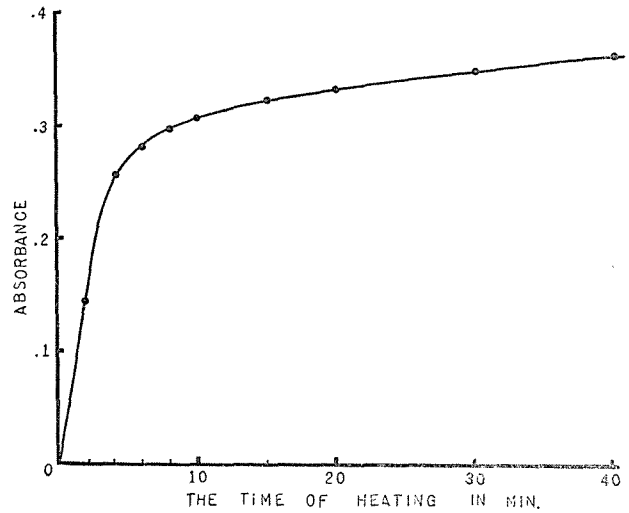

Fig. 2. Relation between the time of heating and absorbance of the developed color.

In this figure, it is seen that the absorbance rapidly increases within the first 10 minutes. This fact means that the Dische reaction is not completed when the mixture is heated for within 10 minutes. It is necessary, therefore, to heat the mixture at least 20 minutes.

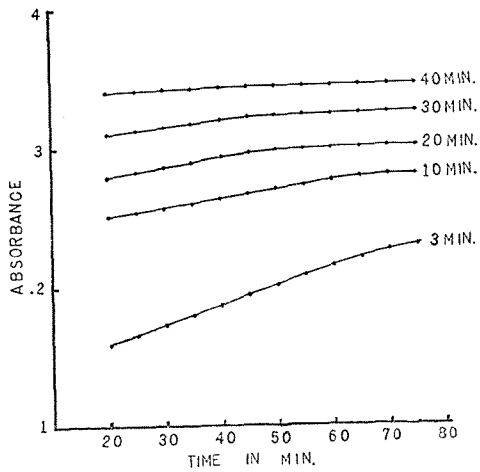

Fig. 3. The changes of absorbance of the developed color after heating. The time of heating is noted in each curve.

Experiment 2. Stability of reaction products.

(a) Fffect of the time of heating. The N/10-KOH solutions containing Na-DNA (0.3mg/ml) were heated for 3, 10, 20, 30 and 40 minutes at 90°C. The changes of absorbance of the developed color after heating was measured. The results obtained are shown in Fig. 3.

In this figure, it is shown that the longer the time of heating the more stable the color developed, but the heating for 30 and 40 minutes should be avoided as the developed color is dark and not typical as stated in Experiment 1. Heating for 20

minutes, therefore, is recommended. The same result was obtained in the case of N/2-KOH solution.

(b) Effects of KOH concentration. From the results of the above experiments, it is concluded that the Dische reaction gives the most reasonable result when the reaction is carried out for 20 minutes at 90°C, but the result obtained in Experiment 2 (a), seems to show that the stability of the color developed depends on concentration of KOH to a certain extent.

In this experiment, therefore, the effect of KOH concentration on the stability of the developed color was studied.

Na-DNA was dissolved in N/10- N/5-, N/2-, N-KOH solutions and distilled water. The Dische reagent was added to these solutions and heated for 20 minutes at 90°C. After the blue color developed, the mixtures were cooled in ice water, and the absorbance was measured every 5 minutes (Fig. 4).

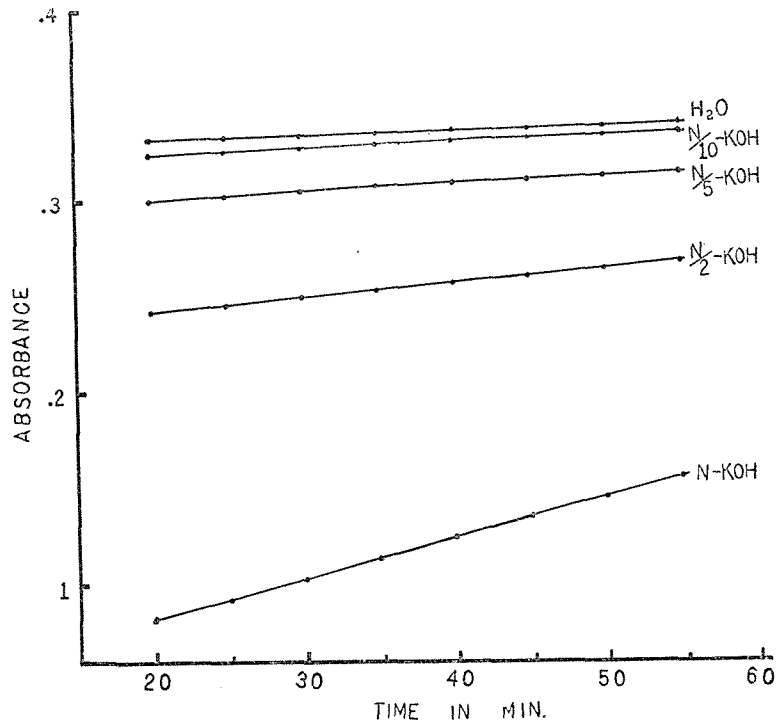

Fig. 4. Relation between the changes of absorbance of the developed color after heating and concentration of KOH soln. Concentration of KOH soln is remarked in every line.

It is seen in Fig. 4 that the development of the reaction color is imcomplete and metastable when the KOH concentration is high so far as the present experi-

ment concerns. That is to say, in the cases of distilled water and N/10-KOH solution, the color is more stable and clear than case of N/5-, N/2- and N-KOH solutions. But as Na-DNA and DNA are only slightly soluble in distilled water, it is not recommended for the solvents of these substances.

Experiment 3. Relation between the absorbance and DNA-concentration.

In this experiment, the relation between Na-DNA concentration and the light absorbed by a layer of colored solution was studied with a Beckman spectrophotometer. The result of the experiment is seen in Fig. 5.

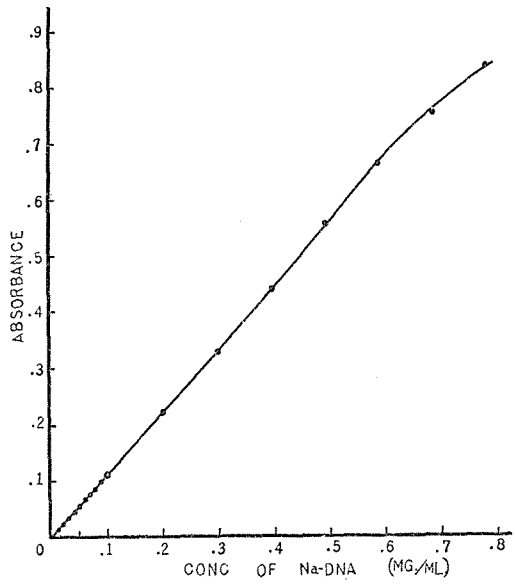

Fig. 5. Relation between the absorbance of the developed color and the concentration of Na-DNA.

In this figure, it is presented that the absorbance increases proportionally to the concentration of DNA at least between 0.01mg/ml and 0.6 mg/ml.

Conclusions

According to the procedure of Dische (1931), the mixtures of DNA and the reagent are heated for 3 minutes in boiling water bath. The result of the present investigation, however, shows that the color developed under this conditions was not stable. In respect to the time of heating, we arrived at the conclusion that the longer the time of heating the color developed was more stable so for as the present investigation concerns. But, if the time of heating is longer than 30 minutes at 90°C the color developed is dark and reddish-blue. This color is hardly regarded as the typical reaction color. (cf. Dische, 1931; Masayama et al, 1940). The result of the present experiment shows that when the mixtures

of DNA and the reagents are heated for 10 and 20 minutes at 90°C, the color developed is clearly blue and stable. It is recommended, therefore, to process for the reaction under the above conditions.

It must be mentioned here that when the reaction was undertaken under the conditions of 38°C-5 hours, 60°C-45 minutes and 60°C-3 hours, the absorbance of the developed color was not proportional to the concentration of DNA.

In respect to the concentration of KOH solution which contains DNA, the results of our experiment show that N/10-KOH solution is preferable to N/5-, N/2- and N-KOH solutions.

If the reaction is carried out under the conditions stated above, that is, Na-DNA is dissolved with N/10-KOH and heated for 20 minutes at 90°C, the quantitative determination of DNA is carried out between 0.01mg/ml and 0.6mg/ml without difficulties.

It is stated here that the experiments reported above were carried out with pure Na-DNA solution, but when proteins and other impurities are present with DNA the quantitative determination is not easily obtained. In respect to this problem, the author wishes to report the results of experiments in elsewhere.

Summary

1. A critical study of Dische reaction for the quantitative determination of DNA was carried out.

2. Wave-length of the maximum absorption of the colored substance developed as a result of the Dische reaction is 600mμ.

3. The results of the model experiments show that the procedure of Dische reaction recommended is as follows :— DNA is dissolved in N/10-KOH solution and heated with the Dische reagent for 20 minutes at 90°C.

4. Under the above conditions, absorbance of the colored substance developed is proportional to DNA concentrations between 0.01mg/ml and 0.6mg/ml.

This work was performed under the direction of Prof. N. Shinke and the author wishes to express his thanks to him for his guidance and valuable criticism.

Literature

Brachet, J., (1950): Chemical Embryology. (New York).

Caspersson, T. O., (1936): Uber den chemischen Aufbau der strukturen des Zellkernes. Skand. Arch. Physiol., Bd. 73, Suppl. Nr. 8.

————————, (1950): Cell Growth and Cell Function. (New York).

Claude, A. and Potter, J. S., (1943): Isolation of Chromatin Threads from the Resting Nucleus of Leukemic Cells. Jl. Exp. Med. Vol. 77.

Dische, Z., (1931): Nachweis und Bestimmung der Thymonukleinsäure. Abderhaldten Handb. d. biolog. Arbeitmeth. Lfg. 355.

Masayama, T., Yokoyama, T. and Schudo, M., (1940): Eine Mikromethode zur Bestimmung der Thymonucleinsäure in tierischen Organen (Japanese). Osaka Igakukai Zasshi., Bd. 39.

Pollister, A. W. and Ris, H., (1947): Nucleoprotein Determination in Cytological preparations. Cold Spring Harbor Symp., Quant. Biol. XII.

Schneider, W. C., (1945): Phosphorus Compounds in Animal Tissues. I. Extraction and Estimation of Desoxypentose Nucleic Acid and Pentose Nucleic Acid. Jl. Biol. Chem., Vol. 169.

Sugiyama, H., Shinke, N. and Ishida, M. R., (1954): A Method of Quantitative Determination of Nucleic Acids in Plant Cells. The Bot. Mag. (Tokyo) Vol. 67.