

tumor induction. Direct chemical measurements of DNA are thus in agreement, not in discord, with histochemical measurements (5), at least for the first 5 or 6 days following infection. DNA changes which do occur in a growing plant following infection with *Agrobacterium tumefaciens* are the result of puncturing and thus also occur in wounded tissues.

The absence of marked quantitative changes in DNA content during initial stages of tumorigenesis does not imply the absence of change in regulatory mechanisms governing DNA synthesis. Our studies, however, do not penetrate to this problem. We may only conclude that if a regulatory change does occur, it is not manifest as an early increase in the rate of DNA synthesis.

LITERATURE CITED

1. BRAUN, A. C. & T. STONIER. 1958. Morphology & physiology of plant tumors. *Protoplasmatologia*. 10 (5a): 1-93.
2. KLEIN, R. M. 1952. Nitrogen & phosphorus fractions, respiration, & structure of normal & crown gall tissues of tomato. *Plant Physiol.* 27: 335-354.
3. KLEIN, R. M., E. M. RASCH, & H. SWIFT. 1953. Nucleic acids and tumor genesis in broad bean. *Cancer Res.* 13: 499-502.
4. KUPILA, S., A. M. BRYAN & H. STERN. 1960. Extractability of DNA & its determination in tissues of higher plants. *Plant Physiol.* 36: 212-215.
5. RASCH, E., H. SWIFT, & R. M. KLEIN. 1959. Nucleoprotein changes in plant tumor growth. *J. Biophys. Biochem. Cyt.* 6: 11-34.
6. ROBSON, H. H., M. A. BUDD, & H. T. YOST. 1959. Comparison of the nucleic acid, total nitrogen, & protein nitrogen levels of normal & tumour tissue under similar growth conditions. *Plant Physiol.* 34: 435-440.
7. STERN, H. 1960. Biochemical sequences in mitosis. In: *Developing Cell Systems & Their Control*, D. Rudnick, ed. Ronald Press, N. Y.

RAPID BIOASSAY FOR KINETIN & KININS USING SENESCING LEAF TISSUE¹

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The isolation and characterization of kinetin (6-furfurylaminopurine) was first reported in 1955 by Miller, Skoog, von Saltza, and Strong (4). Since then both kinetin and a number of its analogs have been shown to have remarkable effects on plant growth (1, 2, 5, 7). So far, however, no natural kinin has been isolated and identified. The search for natural kinins and their subsequent isolation has been hampered by lack of a fast and simple bioassay. To meet this need we have developed the following quantitative assay which is based upon the ability of kinetin to retard the degradation of chlorophyll in the cells of

senescing leaf tissue (7). It is quantitative over the range from 0.05 to 5.0 μ g of kinetin and may be used for detecting still larger amounts, although it is then no longer quantitative. The assay may be used either to test zones cut directly from paper chromatograms or for material in solution. Unlike tests for kinetin based on growth responses (3), the present method does not require the addition of auxin.

MATERIAL & METHODS

Cocklebur plants (*Xanthium Pensylvanicum* Wall.) of the inbred strain used in the California Institute of Technology are grown from seed in a greenhouse (max temp 30° C, min 20° C; air de-smogged with charcoal filters). Normal daylight is supplemented with incandescent light to give a 20 hour photoperiod. Under these conditions the mature leaves are thin and soft in texture and attain an area

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of about 150 cm². When the plants have produced ten leaves in all, the fifth fully-expanded leaf (counting back from the apex) is suitable for the bioassay. The leaves are harvested and stored with the petioles in water under conditions of low light intensity (50 ft-c) for 3 days in Plexiglas boxes at 20° C. During this period, metabolic processes leading to senescence are initiated and there is a fall in the total chlorophyll content of the leaf. The final selection of suitable leaves is made on the basis of a uniform pale green color of the blade.

Kinetin standards are prepared as follows: 0.5 ml of aqueous solutions of kinetin at concentrations ranging from 16 to 0.063 mg/l are applied uniformly to circles of Whatman no. 1 filter paper, 4.25 cm in diameter placed in the base of a 5 cm petri dish. Control circles receive 0.5 ml of distilled water. Paper for the chromatography of extracts must be thoroughly washed to avoid accumulation of toxic materials just behind the solvent front. Ethyl acetate, acetic acid, water, (10:5:2, V/V) (8) is very effective for this purpose. Uniform discs 12 mm in diameter are cut from the interveinal blade tissue of previously aged and selected cocklebur leaves. Some 60 discs may easily be obtained from a suitable leaf. Four discs are placed on each filter-paper circle or portion of chromatogram so that the abaxial surface of the leaf tissue is in contact with the liquid. (0.5 ml is a suitable volume of water to add to portions of chromatogram strip large enough to accommodate four discs.) Duplicate sets of four untreated discs may be extracted immediately in boiling 80 % ethanol and the extract retained for determining the original chlorophyll content of the discs. Each small dish is placed on two layers of damp filter paper inside a larger closed petri dish. The larger dishes are stacked in enamel trays lined with damp filter paper and covered with aluminum foil. They are then maintained in darkness for 48 hours at 24° C. By the end of this period most of the chlorophyll in the controls is degraded and the discs are yellow. Those in the higher kinetin treatments are still green. (If the leaves have been insufficiently aged prior to use the duration of the assay may have to be extended.)

Each group of discs is then dropped into a graduated centrifuge tube containing 5 to 6 ml of hot 80 % ethanol, and boiled gently on a water bath until the chlorophyll has been extracted. The tubes are cooled and the volume made up to 10 ml with 80 % ethanol. The optical density of each solution was measured against 80 % ethanol in a Beckman spectrophotometer. Model B at 665 m μ and 645 m μ , the absorption maxima in the red region for chlorophyll a and chlorophyll b, respectively.

RESULTS

The optical densities of extracts of leaf discs which had been exposed to kinetin standards ranging from 0.031 to 25 μ g are presented in figure 1. It is clear that since the chlorophyll a/chlorophyll b ratio

remains approximately the same in the presence of kinetin the determination of the optical density of the extract at either the chlorophyll a maximum or the chlorophyll b maximum is suitable as a measure of the retention of total chlorophyll in the leaf tissue. However, since it is advantageous to cover a wide range of optical densities, measurement at the wavelength of the maximum absorption of the chlorophyll a (665 m μ) is recommended.

Some absorption at both these wave lengths is found even when all the chlorophyll has disappeared. This is due to the presence of yellow pigments which are not degraded as rapidly as chlorophyll.

The retention of chlorophyll bears a linear relation to the logarithm of the kinetin concentration over a range of approximately 0.05 to 5.0 μ g. These values correspond to concentrations of 0.1 to 10 mg/l. The relationship is not linear for quantities of kinetin

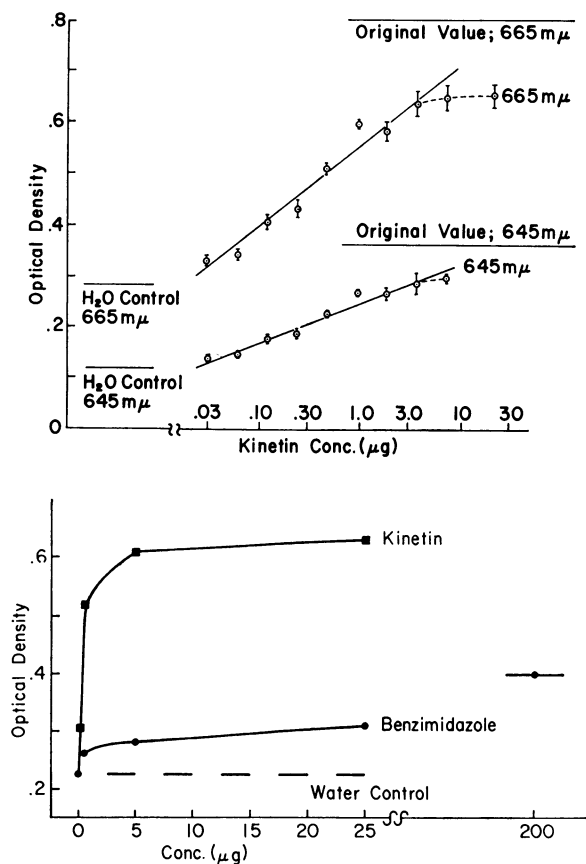


FIG. 1. Retention of chlorophyll by kinetin in discs of senescing Xanthium leaves. Optical densities of chlorophyll extracts in 80 % ethanol determined at the chlorophyll a and chlorophyll b maxima 48 hours after treatment.

FIG. 2. Comparison of the effectiveness of benzimidazole and kinetin in preventing loss of chlorophyll in senescing Xanthium leaf discs. Optical density of 80 % ethanol extract determined at 665 m μ .

above 5 μg because a maximum value for chlorophyll retention is obtained. Under these conditions the assay still provides a qualitative test for kinetin at least up to the maximum amount tested (25 μg).

6-Benzylaminopurine which is active in promoting cell divisions in tobacco pith cultures (9) is also active in the leaf disc assay, as is its riboside. Person et al (6) have shown that chlorophyll degradation in detached wheat leaves is prevented by benzimidazole as well as by kinetin. Figure 2 shows that with *Xanthium* leaf discs benzimidazole is not nearly so effective as kinetin.

A number of other compounds have been tested for possible interference in this assay. These include auxins (IAA & 2,4-D) and amino acids which are inactive, purines (adenine, adenosine, & guanosine) and 1,3-dimethylurea which interfere slightly but even at high concentrations give values lower than those obtained with 0.05 μg kinetin. Sugars in large amounts (2 mg) give values approaching those for 0.05 μg kinetin.

DISCUSSION

This bioassay offers a rapid and simple screening test for material with kinetin-like properties. If desired, materials giving positive results in this assay could be tested using the tissue culture techniques. The leaf disc assay has several advantages. It does not require the use of complex media and sterile conditions and it gives quantitative results very rapidly.

LITERATURE CITED

1. KURAISHI, S. 1959. Effect of kinetin analogs on leaf growth. *Sci. Papers, College of Gen. Educatn., Univ. Tokyo* 9: 67-104.
2. MILLER, C. O. 1958. The relationship of the kinetin and red-light promotions of lettuce seed germination. *Plant Physiol.* 33: 115-117.
3. MILLER, C. O. 1960. An assay for kinetin-like materials. *Plant Physiol. suppl.* 35: xxvi.
4. MILLER, C. O., F. SKOOG, M. H. VON SALTZA, & F. M. STRONG. 1955. Kinetin, a cell division factor from deoxyribonucleic acid. *J. Am. Chem. Soc.* 77: 1392.
5. PATAU, K., K. N. DAS, & F. SKOOG. 1957. Induction of DNA synthesis by kinetin & indoleacetic acid in excised tobacco pith tissue. *Physiol. Plantarum* 10: 949-966.
6. PERSON, C., D. J. SAMBORSKI, & F. R. FORSYTH. 1957. Effect of benzimidazole on detached wheat leaves. *Nature* 180: 1294-1295.
7. RICHMOND, A. E. & A. LANG. 1957. Effect of kinetin on protein content & survival of detached *Xanthium* leaves. *Science* 125: 650-651.
8. ROMBERGER, J. A. 1960. A suggested method for the fractionation of plant extracts. *Pacific Southwest Forest & Range Experiment Station, Berkeley, Cal.* P. 9.
9. STRONG, F. M. 1958. *Topics in microbial chemistry.* John Wiley & Sons, New York. P. 125.