

Effect of Infection with Southern Bean Mosaic Virus and the Diurnal Cycle on the Free Nucleotide Pool of Bean Leaves

R. F. Bozarth and R. E. Browning

Associate Virologist and Research Assistant, Boyce Thompson Institute for Plant Research, Yonkers, New York 10701.

Supported in part by Grant No. CA 04294 from the U.S. Public Health Service and by the Margaret Thompson Biddle Foundation.

Accepted for publication 15 December 1969.

ABSTRACT

Analysis of the acid-soluble nucleotides of fully expanded primary bean leaves and similar leaves previously inoculated with southern bean mosaic virus indicated the presence of 21 organic phosphorus compounds, 11 of which were present in sufficient concentration for identification and quantitative analysis.

The adenosine triphosphate concentration of infected and normal leaves varied in a similar and apparently cyclic manner with lowest quantities during the light and highest quantities during the dark periods. A higher concentration was found in diseased tissue at all times of sampling. *Phytopathology* 60:852-855.

Previous results have indicated that virus infection has significant effects on the quantities of acid-soluble nucleotides in infected tissues. Sunderland & Merrett (13, 14, 15) reported significant changes in pyridine nucleotides and adenosine nucleotides in tobacco mosaic virus-infected tobacco leaves and tissue culture. Bozarth & Browning (2) reported that bean plants infected with southern bean mosaic virus (SBMV) had a significantly higher ATP level and lower GTP level than control plants.

In the latter study, leaf samples were harvested from greenhouse-grown plants in the early afternoon. The differences in amount of nucleotides, although significant, were not great. It is possible that other factors may have a greater bearing on free nucleotides than virus infection, particularly the stress imposed by photosynthesis and respiration throughout the diurnal cycle of light and dark. In the following study, therefore, inoculated leaves of bean plants were harvested at 4-hr intervals during the time of maximum virus synthesis. The plants were maintained in a growth chamber and subjected to 12 hr of darkness and 12 hr of light.

MATERIALS AND METHODS.—Darco-KB charcoal (Atlas Chemical Corp., Wilmington, Delaware) was twice boiled in solution of ammonia (28%), ethanol, and water (1:50:49), washed with water on a Büchner funnel, twice boiled with 6 N HCl, drained, washed with H₂O, and dried overnight at 100 C (8). Celite-545 and analytical-grade Celite were obtained from Johns Manville Corp., New York, N.Y. Bean plants (*Phaseolus vulgaris* L. 'Bountiful') growing in 4-inch pots were selected for uniformity and thinned to five plants/pot 8 days after seeding when the primary leaves were fully expanded. Test plants were inoculated with a suspension of SBMV (0.4 mg/ml) in 0.05 M phosphate buffer using Carborundum as an abrasive and cotton swabs to rub the leaves. Control plants were treated similarly, except that they were rubbed with buffer. The plants were rinsed and placed in a growth chamber maintained at 24 C on a cycle of 12 hr of darkness and 12 hr of light. Illumination was approximately 1,000 ft.

The first harvest was made 30 min after illumination on the 5th day; subsequent harvests were made at

4-hr intervals thereafter for a total of 24 hr. Three replicate samples were taken at each harvest. Each sample consisted of the two primary leaves of 15 plants from three pots selected at random from the growth chamber. Harvests during the dark period were made with the aid of a green light. A sample for bioassay of virus titer was made by punching a 1-cm cork borer through the stack of 30 leaves before weighing the leaves. The sample for bioassay was frozen until a later date. The leaves were immediately plunged into a Waring Blendor containing 200 ml of 5% trichloroacetic acid (TCA) and 20 g of Celite at 4 C and blended for 1 min. The elapsed time between the beginning of harvest of a single replicate sample and the killing of the tissue by blending in the first step of the nucleotide extraction was less than 2 min. The six inoculated and control samples were harvested alternately over a 20-min period. The nucleotide extraction procedure was carried out immediately, with all operations performed in a cold room at 4 C.

After all six samples had been harvested, they were blended for an additional 5 min. The suspension was filtered, using a pad of 5-g Celite-545 on a filter paper in a 9-cm Büchner funnel. The residue and filter pad were returned to the Waring Blendor; 200 ml of a 5% TCA solution was added, and the mixture was blended for 3 min and filtered as above. This step was repeated to yield a clear filtrate. Three 0.6-g portions of Darco-KB were added at 20-min intervals to the combined filtrates while stirring continuously. Twenty min after the last Darco-KB aliquot was added, 40 g of Celite-545 were stirred in and the suspension was filtered in a 9-cm Büchner funnel with a preformed three-layer pad of Celite built up on a sheet of filter paper as follows: 5 g of Celite-545, then 2 g analytical Celite, then 5 g of Celite-545 were used in the three-layer filter bed. The residue was washed with 600 ml water, then suspended in 400 ml of a solution of diethylamine-ethanol-water (14:200:786 v/v), and stirred for 60 min. The suspension was filtered in a 9-cm Büchner funnel on a three-layer pad consisting of 10 g Celite-545, 2 g analytical Celite, and 10 g Celite-545. The residue was washed with two 100-ml portions of diethylamine solutions, and the filtrate was stored in a 1-liter poly-

propylene bottle at -20°C . For column chromatography, the solution was evaporated to dryness in a flash evaporator and the residue was dissolved in 10 ml of deionized H_2O containing 1 drop of concentrated NH_4OH , centrifuged to remove any precipitate, and either stored at -20°C or loaded immediately onto a column of Dowex 1-X8 formate (20 to $40\ \mu$) (Dow Chemical Co., Midland, Mich.) for chromatography.

Nucleotides were analyzed directly in column effluents by a modification of the automated phosphorus technique of Weinstein et al. (16). The modifications were as follows [the letters in parentheses refer to Fig. 1 of the previously published flow diagram (17)]: Flow rate through the column (a) was 1.6 ml/min. Of this, 1.2 ml/min (b) was mixed with 50% sulfuric acid, pumping at the rate of 1.20 ml/min (c); the rest was diverted to a fraction collector. The water dilution (g) was omitted and the sample was segmented with air at 0.42 ml/min.

The digested sample from the impinger was pumped at 0.42 ml/min (i), diluted with water pumped at 0.42 ml/min (m), and mixed with 1.5% ammonium molybdate pumped at 0.60 ml/min (o) and 1-amino-2-naphtholsulfonic acid at 0.80 ml/min (p). Other quantities remained the same.

The modified analytical procedure resulted in increased sensitivity such that a sample equivalent to about 1 g fresh wt of tissue was applied to each Dowex column. Quantitative measurements of nucleotide peaks were made by planimetry and related to curves produced from standard nucleotides. Abbreviations used throughout the text are: CMP, CDP, CTP, AMP, ADP, ATP, GMP, GDP, GTP, UMP, UDP, and UTP for the respective mono-, di-, and trinucleotides of cytidine, adenine, guanosine, and uridine; NAD and NADP for nicotinamide dinucleotide, and its dinucleotide phosphate; ADPR and ATPR for adenosine di- and triphosphoribose; and UDPG for uridine diphosphoglucose.

In preliminary experiments in which the method was tested in pea tissue and percentage recovery was estimated by the addition of labeled nucleotides to extraction mixtures, percentage recovery of five nucleotides was estimated to be: UMP, 35%; CTP, 65%; ATP, 64%; UTP, 51%; and GTP, 72%. A small proportion of the triphosphates was hydrolyzed to the respective mono- and diphosphates.

Virus titer was determined by bioassay of samples on bean (*Phaseolus vulgaris* 'Pinto') using the two dilution assay techniques of Spencer & Price (12).

RESULTS.—The means and standard deviations of the analysis of 11 nucleotide phosphates are shown in Fig. 1. The nucleotides were present in concentrations of about 0.5 to $2\ \mu\text{g/g}$ fresh wt of tissue.

The ATP concentration changed throughout the experiment in a manner that appeared to be cyclic. It dropped to the lowest quantity for the experiment 4.5 hr after lights were turned on, and rose to the highest value 4.5 hr after lights were turned off. Infected and normal tissues had similar cyclic patterns, but infected tissue consistently had a higher ATP content.

UMP appears to have different cyclic patterns for

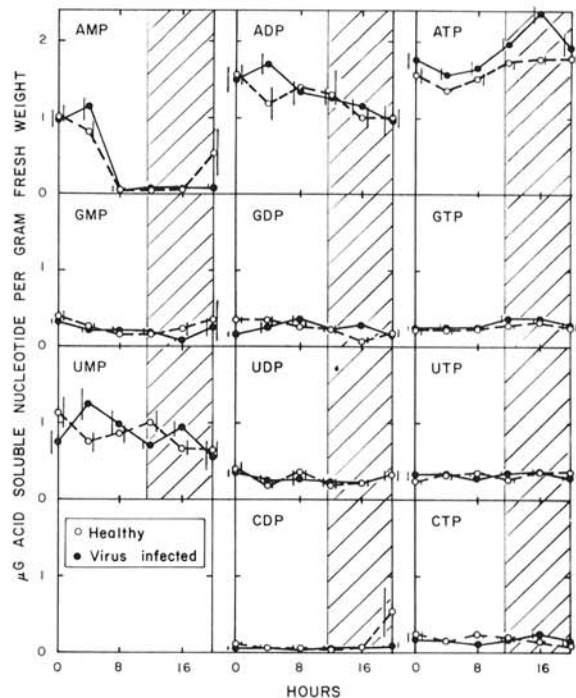


Fig. 1. Concentration of acid soluble nucleotides in normal (broken line) and southern bean mosaic virus-infected (solid line) bean leaves at 4-hr intervals beginning 30 min after lights were turned on on the 5th day after inoculation. The first three samples were harvested during the 12-hr light period (open background); the second three samples were harvested during the 12-hr dark period which followed (lined background). The vertical bars indicate the standard deviations of the sample means ($n = 3$).

diseased and normal tissue. AMP and ADP generally decreased throughout the experiment, and at certain times of sampling there appeared to be a difference between the concentration in diseased and normal tissue. Other nucleotides, GMP, GDP, GTP, UDP, UTP, CDP, and CTP appear to be unaffected by diurnal cycle of light and dark or by virus infection. CMP was not detected in any tissue.

Ten additional phosphorus-containing peaks occurred regularly, but because of their extremely low concentration were only tentatively identified. Among these were peaks considered to be ADPR and ATPR, hydrolysis products of NAD and NADP (7), and UDPG. There was no consistent effect of diurnal cycle or virus infection on any of these 10 phosphorus-containing substances.

Bioassay of samples indicated that the leaves were infected, but the assay procedures used were not sufficiently precise to detect a change in titer during the 24-hr sampling period of the experiment.

DISCUSSION.—Either growth or drastic changes in environment can result in significant changes in the free nucleotide or pyridine nucleotide pools of plants. Brown (4) reported a 250% increase in ATP and a parallel fall in AMP following germination of pea seedlings. Wilson & Harris (19) reported rapid syntheses of ATP and UDPG in crested wheat grass seeds during

the first 6 hr of imbibition. Cherry & Hageman (6) reported that the age of tissue affects the nucleotide composition of etiolated corn seedlings. Pakhomova et al. (9) noted large changes in several nucleotides between algae cells cultured in the dark and in the light. West (18) reported that water relations affect the nucleotide composition of plants. Yamamoto (21) considered the level of the pyridine nucleotides to be the controlling factor in carbohydrate metabolism of plant tissue. A 4- to 5-fold increase in purines in wheat seedlings infected with *Puccinia graminis tritici* was reported by Shaw & Srivastava (10).

The effect of virus infection on acid-soluble nucleotides in plant tissues has been studied only by Sunderland & Merrett (13, 14, 15) and Bozarth & Browning (2). The former authors obtained different results in tobacco callus tissue culture from those obtained with inoculated leaves of a local-lesion host to tobacco mosaic virus. Infected tissue cultures had lower ADP and ATP concentrations, whereas infected leaves had higher ADP and ATP concentrations. Similar anomalies were also found in their analyses of pyridine nucleotides. They suggested that the nucleotide levels observed were correlated with increased respiration reported for infected leaves and decreased respiration previously observed in systemically infected tobacco plants.

In this study, the ATP concentration was consistently higher in diseased than in healthy bean leaves. The difference was not great, but it was consistent at all times of harvest. ADP, on the other hand, was equal in healthy and diseased tissue at four of six times of sampling, and there was no suggestion of the diurnal cycle observed in the case of ATP.

The observed diurnal cycle of ATP concentration follows the well-known acidity cycle observed in many plants (1, 11). It was somewhat surprising that the ATP concentration was lowest during the daylight, but there is some precedent for this observation in the work of Pakhomova et al. (9) who reported that ATP was absent in the nucleotide pool of algae grown in the light but present in algae exposed to darkness.

Brown (4) noted an apparently close correlation between ATP production and utilization, and pointed out that the quantity of free nucleotides might be an indication of the amount of metabolic activity (3). The increased quantity of ATP in virus-infected leaves probably reflects only a more active metabolism as in the case of TMV-infected *Nicotiana glutinosa* leaves (15).

Other than those changes in ATP concentration brought by virus infection, the most striking feature of Fig. 1 is the similarity of nucleotide pools in virus-infected and normal leaves. None of the changes observed is great, and they suggest only slightly altered pool sizes at best. No qualitative changes in nucleotide pools were observed, whereas gross changes in nucleotide and pyridine nucleotide pools had been observed previously in plants undergoing morphogenic changes as in the case of germinating seed (4) or maturation of leaves (21). Wimpenny (20) observed that the nucleotide pool size of bacteria was remarkably constant

unless the cells were disrupted. Bucher & Swaffield (5) also observed that the ATP/ADP ratio of intact rat liver was constant during prolonged anesthesia in the presence of 100% oxygen.

Unfortunately, the number of infected cells is not known. Even during the period of log increase in virus, different cells are at different stages in virus multiplication. The small changes noted may be merely a suggestion of large changes that would be observable if only the infected cells could be included in the sample. Despite the high concentration of SBMV used in the inoculation, there are probably more healthy than infected cells. Thus, the problem of compartmentation which has bothered plant physiologists continuously was also a serious problem in this study.

Taken as a whole, the nucleotide pools of inoculated bean leaves are not greatly affected by SBMV infection.

LITERATURE CITED

- BENNET-CLARK, T. A. 1933. The role of organic acids in plant metabolism, I. *New Phytol.* 32:37-71.
- BOZARTH, R. F., & R. E. BROWNING. 1966. Free nucleotides of southern bean mosaic virus-infected bean leaves. *Phytopathology* 56:871 (Abstr.).
- BROWN, E. G. 1962. The acid-soluble nucleotides of mature pea seeds. *Biochem. J.* 85:633-640.
- BROWN, E. G. 1965. Changes in the free nucleotide and nucleoside pattern of pea seeds in relation to germination. *Biochem. J.* 95:509-514.
- BUCHER, N. R., & M. N. SWAFFIELD. 1966. Nucleotide pools and [6-¹⁴C] orotic acid incorporation in early regenerating rat liver. *Biochim. Biophys. Acta* 129:445-459.
- CHERRY, J. H., & R. H. HAGEMAN. 1960. Separation and identification of soluble nucleotides from etiolated corn seedlings as a function of growth. *Plant Physiol.* 35:343-352.
- FORREST, R. J., D. R. WILKEN, & R. G. HANSEN. 1960. The presence of adenosine diphosphoribose and adenosine triphosphoribose in acid extracts of certain animal tissues. *Biochim. Biophys. Acta* 37:551-553.
- HURST, R. O., & G. C. BECKLING. 1963. Hydrolysis of calf thymus deoxyribonucleate by pancreatic deoxyribonuclease. I. A study of the oligonucleotides liberated in the presence of magnesium or manganese ions. *Can. J. Biochem. Physiol.* 41:469-480.
- PAKHOMOVA, M. V., G. T. DARKANBAEVA, & G. N. ZAITSEVA. 1966. Effect of light and darkness on the content of acid-soluble phosphorus compounds in the green alga *Scenedesmus obliquus* Kütz. *Biochemistry (USSR) (English Translation)* 31:1065-1072.
- SHAW, M., & B. I. S. SRIVASTAVA. 1964. The physiology of host-parasite relations, XIII. The effect of stem rust on purines in wheat leaves. *Can. J. Bot.* 42:139-144.
- SIDERIS, C. P., H. Y. YOUNG, & H. H. Q. CHUN. 1948. Diurnal changes and growth rates as associated with ascorbic acid, titratable acidity, carbohydrate and nitrogenous fractions in the leaves of *Ananas comosus* (L.) Merr. *Plant Physiol.* 23:38-69.
- SPENCER, E. L., & W. C. PRICE. 1943. Accuracy of the local-lesion method for measuring virus activity, I. Tobacco-mosaic virus. *Amer. J. Bot.* 30:280-290.
- SUNDERLAND, D. W., & M. J. MERRETT. 1963. Nicotinamide-dinucleotides, adenosine diphosphate and adenosine triphosphate content of tissues infected by tobacco mosaic virus. *Nature* 199:1116-1117.
- SUNDERLAND, D. W., & M. J. MERRETT. 1963. Nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate concentrations in leaves of *Nicotiana glutinosa* infected by tobacco mosaic virus. *Nature* 200:921.

15. SUNDERLAND, D. W., & M. J. MERRETT. 1964. Adenosine diphosphate and adenosine triphosphate concentrations in leaves showing necrotic local virus lesions. *Virology* 23:274-276.
16. WEINSTEIN, L. H., R. F. BOZARTH, R. H. MANDL, S. GROSS, & J. F. GOLDMAN. 1965. Automated analysis of phosphorus-containing compounds in biological materials. II. Acid-soluble nucleotides. *Anal. Biochem.* 11:155-158.
17. WEINSTEIN, L. H., R. F. BOZARTH, C. A. PORTER, R. H. MANDL, & B. G. TWEEDY. 1964. Automated analysis of phosphorus containing compounds in biological materials, I. A quantitative procedure. *Contrib. Boyce Thompson Inst.* 22:389-397.
18. WEST, S. H. 1962. Protein, nucleotide, and ribonucleic acid metabolism in corn during germination under water stress. *Plant Physiol.* 37:565-571.
19. WILSON, A. M., & G. A. HARRIS. 1966. Hexose-, inositol-, and nucleoside phosphate esters in germinating seeds of crested wheatgrass. *Plant Physiol.* 41:1416-1419.
20. WIMPENNY, J. W. T. 1967. Adenosine triphosphate pool levels and turnover in micro-organisms. *Biochem. J.* 102:34P-35P.
21. YAMAMOTO, Y. 1963. Pyridine nucleotide content in the higher plant. Effect of age of tissue. *Plant Physiol.* 38:45-54.