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# Effects of Cytokinins on the Respiration of Soybean Callus Tissue[1]

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## ABSTRACT

**A technique which incorporates a brief blending step to disperse callus tissue into small clumps of cells was developed, and the effects of cytokinins on respiration of soybean (*Glycine max* [L.] Merrill var. Acme) callus tissue prepared in this way were studied. Adenine alone did not affect respiration, but kinetin and zeatin showed effects correlating with their reported effects on growth of this tissue; after about 3 hours both hormones promoted respiration at concentrations which promote growth, while kinetin, but not zeatin, also exhibited inhibition at higher concentrations. Studies with 2,4-dinitrophenol led to the suggestion that although the respiration of this tissue is largely under the control of ATP levels, kinetin does not exert its control on respiration through effects on ATP levels or oxidative phosphorylation during the monitoring period. Further inhibitor and substrate studies provided evidence that the promotion of respiration by kinetin results from an increase in substrate entering the tricarboxylic acid cycle, perhaps by an effect on pyruvate metabolism.**

**The inhibition of respiration by high concentrations of kinetin is partially due to effects on oxidative phosphorylation or ATP utilization, but 70% of the inhibition cannot be attributed to this.**

Cytokinins promote, or inhibit, oxygen uptake and carbon dioxide evolution in a variety of plant tissues (3, 11). Explanations of these effects are diverse and little attempt has been made to correlate them with other cytokinin effects in the tissue used, or even to show that they are specific cytokinin responses. In this laboratory, soybean callus tissue is used routinely in cytokinin assays (5), so its growth responses to cytokinins is well known. Furthermore, some progress has been made in describing its early biochemical responses to the compounds (6, 7). With this background of information, a study of the respiration of the tissue could help to explain some of the cytokinin effects and to indicate relationships of the effects to each other. We have found that, depending on the concentration, either a promotion or an inhibition of oxygen uptake by kinetin occurs. Similarities between the respiratory responses to cytokinins and known growth responses are discussed herein and preliminary attempts to define the respiratory effects are presented.

## MATERIALS AND METHODS

**Maintenance of Stock Cultures.** All the experiments were performed with *Glycine max* (L.) Merrill var. Acme callus tissue. Stocks were subcultured from parent cultures originally derived from cotyledons and grown at 27 ± 1 C and about 300 lux fluorescent light (5). Flasks were rotated frequently on the shelves in order to assure about the same amount of light exposure by the time of use. Tissue was used 40 to 50 days after subculturing.

**Preparation of Tissue for Experiments.** All operations were in a 4 C room with materials and solutions, except as indicated, chilled to that temperature. The tissue was not prechilled. Blending, suspension, and wash solutions were the same as the experimental control medium without cytokinin. For some experiments all media and utensils were sterilized, whereas for others they were not; the sterilization did not influence the results.

About 25 to 30 g of callus tissue was added to 100 ml of blending medium in a stainless steel, 250-ml Waring Blendor chamber. The tissue was blended for 12 to 15 sec at low speed with a Model PB-5 Waring Blendor. The resulting suspension was filtered through a piece of Miracloth (Calbiochem) in a Büchner funnel with a partial vacuum provided by a two-way rubber suction bulb. The tissue was washed by sucking 100 ml of the same medium through it, resuspended in 100 ml of fresh medium, filtered onto a fresh piece of Miracloth, and washed as before with a further 200 ml of medium. Excess liquid was removed by suction.

The tissue was then transferred to a Petri plate and mixed with a stainless steel spatula in order to break the mass into small, easily-handled clumps. For most experiments, 1.0-g portions of the tissue were removed from the plate and added to 25 ml of medium in each of the 50-ml treatment flasks. Since cytokinin effects became evident only some time after addition of the hormone (see later), an incubation period on a shaker preceded the actual oxygen measurements. A precise time schedule was followed so that the interval between the start of the experimental treatment, and the start of measuring the oxygen uptake was the same in all samples. This was necessary because of the respiratory drift evident in Figure 1; this drift begins only after the tissue is removed from the cold at the beginning of the incubation period.

**Treatment of the Tissue.** The tissue was incubated in media containing 0.1 M sucrose (as normally used in the growth medium) or 0.1 M mannitol with 10 mM potassium biphthalate buffer for pH 4.5 and 5.0 or potassium phosphate for pH 5.8

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and above. MES[3] buffer at pH 6.0 was used in one experiment. The pH was adjusted with KOH except where sodium was substituted for potassium in which case NaOH was used. Little or no change in pH was found over the course of the experiments. Kinetin, when supplied, was used at 1 $\mu$M unless otherwise indicated. The flasks were maintained at room temperature and only briefly exposed to the cold while adding the tissue. The samples were shaken for various periods on a New Brunswick gyratory shaker Model S-3 at 160 cpm. Unless noted otherwise, the shaking period before the oxygen measurement was 4 hr. Light and temperature were the same as for growth of the stock cultures. Three or four replicates were used for each treatment.

For the various inhibitor experiments tissue was shaken in 24 ml of medium for 2 hr and then 1 ml of concentrated inhibitor solution was added. An additional 2 hr of shaking completed the 4 hr total. For the substrate addition experiments, the substrate was included in the medium from the beginning. Inhibitor experiments were performed at pH 4.5 (except DIECA and KCN which were used at pH 7) while substrates, except where indicated for pyruvate, were used at pH 5.8. Sodium salts were used, except for KCN.

**Oxygen Electrode Measurements.** A Yellow Springs Instrument Co. Model 53 biological oxygen monitor was used to measure $O_2$ uptake. This was equipped with a Clark electrode, a Haake Model FE constant temperature circulator to maintain the sample chamber temperature (27 C), and a Hewlett-Packard Moseley Model 680M strip chart recorder. Stirring discs provided with the instrument were replaced with 1.5-cm magnetic bars coated with Teflon in order to reduce tissue destruction in the measuring chamber.

The samples of treated tissue were read in the order of original preparation and at equal time intervals. After the shaking period the contents of the flasks were poured onto Miracloth in a Büchner funnel. Remaining cells were washed onto the Miracloth by rinsing the flask twice. Extra medium was forced through with a slight vacuum provided by an aspirator. The cells were then scraped off the Miracloth, placed into 10 ml of fresh treatment medium in the sample chamber, and the temperature was equilibrated for 3 min while stirring and bubbling water-saturated air through the cell suspension. The electrode was calibrated to 100% with air-saturated water and measurements were made for 4 to 5 min. A value of 0.25 $\mu$mole $O_2$/ml for water saturated with air at 27 C was used for the calculations (calculated from 2).

The time course of respiration changes at pH 4.5 and 7.0 was measured by continually rotating one electrode from chamber to chamber; three chambers contained tissue added immediately after blending and washing (no shaking period was used) while the fourth contained air-saturated water in order to correct for instrument drift if necessary (drift was usually less than 1%). Measurements were made for 2- to 3-min periods on each chamber in succession. When $O_2$ uptake was not being measured in a given chamber, the stirring bar was removed and water-saturated air was bubbled through it.

In the short term addition experiments with 2,4-DNP, the tissue was treated for 4 hr with or without kinetin and then a respiratory baseline was read for about 4 to 5 min. The uncoupler was then added directly to the test chamber through a notch in the side of the electrode while continually monitoring $O_2$ uptake and readings were made for an additional 5 to 6 min.

[3] Abbreviations: CCCP: carbonylcyanide-*m*-chlorophenylhydrazone; DIECA: diethyldithiocarbamate; 2,4-DNP: 2,4-dinitrophenol; KPI: kinetin-promotion-increment; MES: 2-N(morpholino)ethanesulfonic acid.

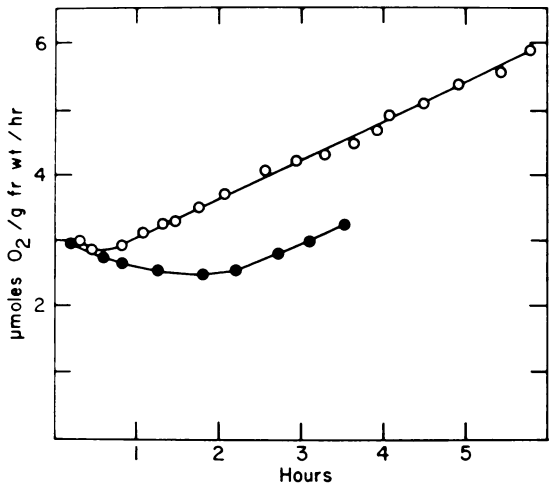


FIG. 1. Respiration rate *versus* time of incubation. Tissue added to medium at pH 4.5 (●) and pH 7.0 (○).

## RESULTS

**Assay Conditions.** The tissue preparation procedure described here produces small tissue clumps which allow ready diffusion of oxygen, metabolites, or inhibitors into the cells; these are factors which produce many uncertainties in tissue slice techniques. In addition, the cells are easily manipulated and treated. However, the effects of the brief blending on the cells (although microscopic studies assured that essentially all the broken cells were removed by the filtration) and the degree of loss of metabolic intermediates upon addition of the cells to fresh medium are unknown.

Figure 1 shows respiration rate changes with increasing incubation times. The rate of $O_2$ uptake initially decreased, but a steady, linear rise followed. The degree and duration of the initial decrease in activity were pH dependent, a lower pH resulting in a greater, more enduring decrease. These data emphasize the need for a precise time schedule when comparing treatments. They also demonstrate the reason the basal respiratory rates in different experiments with different medium pH values are not the same; a second reason for some of the variability in basal rates between experiments is the variable removal of excess medium from the tissue prior to weighing. It should be emphasized, however, that the variability between replicates within each experiment was very low.

**Cytokinin Response Time Course.** In experiments of less than 1 hr, 1 $\mu$M kinetin caused no measurable difference in respiration rates. Frequently, however, kinetin promoted respiration in 2 hr with the increment of promotion further increasing by 3 hr (Fig. 2A). Still longer periods (Fig. 2B) showed that this increasing interval extended to at least 8 hr at which time the basal rate began to level off. This particular long term study also demonstrated an early kinetin inhibition of respiration at the 2-hr reading, a response with variable timing which occasionally occurred as late as 4 hr after the beginning of the experiment. Its occurrence that late was rare, however, and promotion generally was exhibited at 4 hr, so this treatment period was used for the remainder of the experiments.

**Cytokinin Concentration Series.** The specificity of the reaction was determined by comparing identical concentrations of kinetin, zeatin, and adenine (Fig. 3). Adenine did not affect respiration at any concentration used, while both kinetin and zeatin promoted respiration at concentrations as low as 0.05 $\mu$M. In addition, kinetin effected a loss of this promotion at 10 $\mu$M and inhibited respiration approximately 20% at 50 $\mu$M. Zeatin,

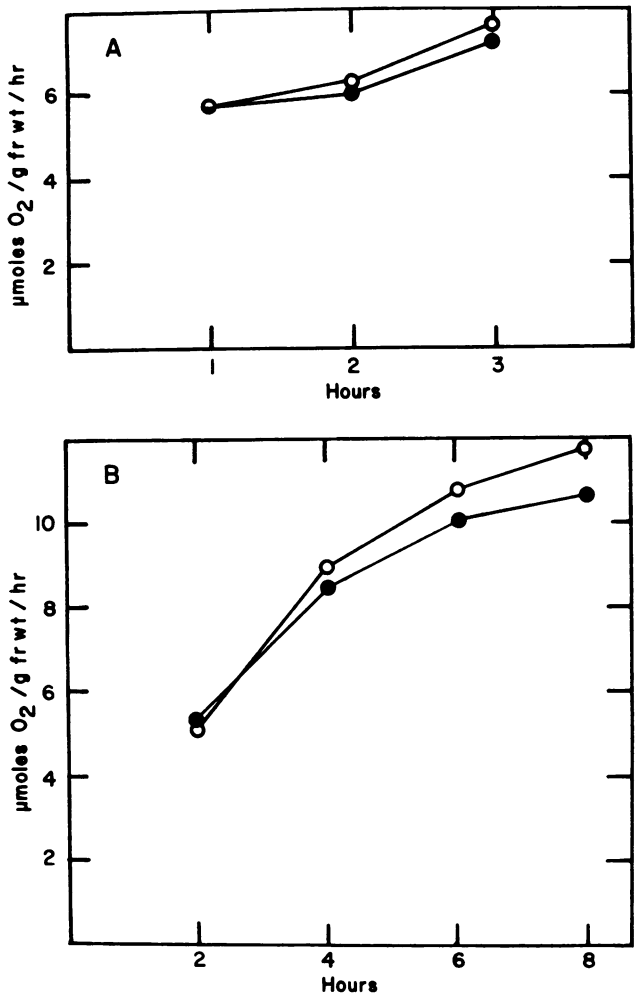


FIG. 2. Changes with time in respiration rates of untreated (●) and 1 µM kinetin-treated (○) tissue. A: Short term; B: long term (see text for discussion of early inhibition). Each point represents an average of four replicates and all points have a standard deviation of less than ±0.08.

however, gave its highest promotion at both 10 µM and 50 µM. The peak of kinetin response appears to lie between 1 and 5 µM, while that for zeatin, if it exists, is at a higher undetermined concentration.

**Effect of Medium Components.** The influence of components of the incubation medium on the kinetin respiratory promotion was investigated by substituting mannitol for sucrose or MES buffer adjusted with NaOH for potassium phosphate. The basal respiratory rate with sucrose and phosphate buffer was 10.23 µmoles $O_2$/g fresh weight·hr, while with mannitol this was reduced to 7.81; 1 µM kinetin increased these values to 10.80 and 8.31, respectively. Use of MES in place of phosphate with no potassium present gave a basal rate of 7.71 or 8.15 with 4 mM KCl also present. Here, kinetin stimulated respiration to 8.24 and 8.61 µmoles $O_2$/g fresh weight·hr, respectively. In short, kinetin promoted respiration even when the usual components of the medium, sucrose, potassium, and phosphate, were missing.

**Preliminary Definition of Cytokinin Promotion.** Inhibitors were used to further define the promotion effect of kinetin; maximum inhibitory concentrations were not used in most cases in order to minimize side effects. Results with cyanide, fluoroacetate, iodoacetate, and NaF (Table I) all indicated that these inhibitors prevented at least part of the increase in oxygen uptake brought about by kinetin. A more detailed study with iodoacetate (Table II) showed that increasing the concentration of this inhibitor further decreased the KPI and a high concentration eliminated the increment entirely. Sodium fluoride gave similar results. 2,4-DNP did not affect the KPI even though a 5 µM concentration promoted respiration about 60% at pH 4.5. It should be recalled here that 2,4-DNP was added after kinetin had a chance to operate. No effect of copper enzyme inhibitors, phenylthiourea and DIECA, on the KPI was found.

The addition of certain substrates affected the KPI. Acetate, malate, and succinate all increased respiration of the control tissue more than they did that of kinetin-treated material (Table III). Succinate, tested at 1 and 3 mM, clearly showed a decreasing KPI with increasing concentrations. In contrast, 10 mM pyruvate did not affect the KPI, even though it did promote respiration in both kinetin-treated and untreated tissue.

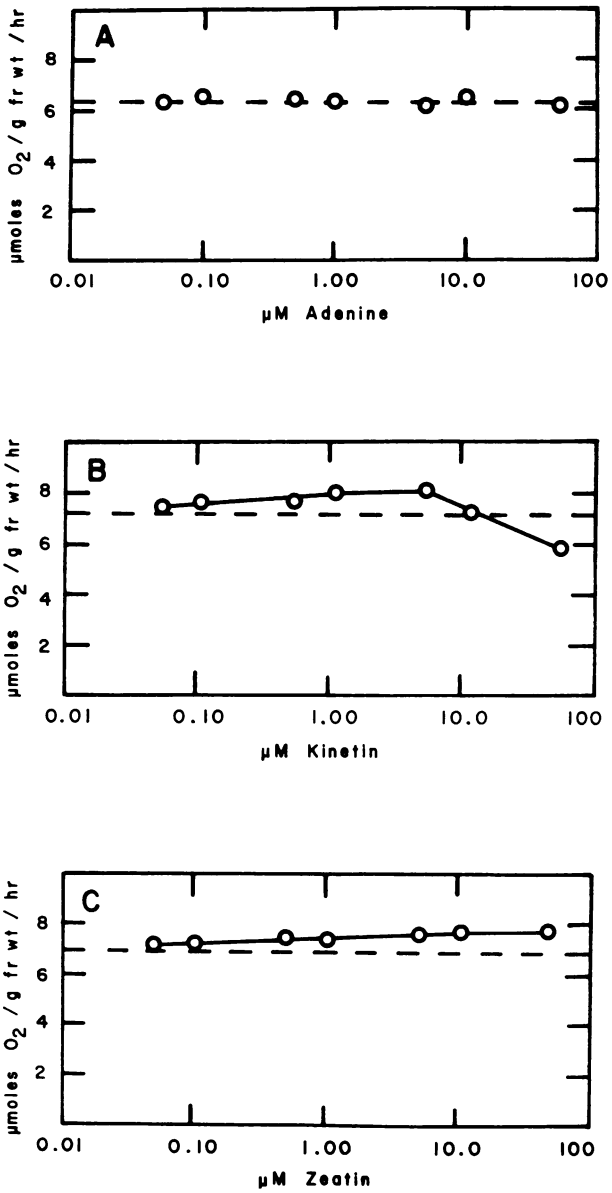


FIG. 3. Respiration rate *versus* adenine or cytokinin concentration: A: adenine; B: kinetin; C: zeatin. Rates measured after 4 hr shaking. Dashed lines represent the control value. Each point represents averages of three replicates.

Table I. *Effect of Various Inhibitors on the Respiration of Soybean Tissue and on the Kinetin Promotion Increment*

| Treatment | Rate of Respiration | | |
|---|---|---|---|
| | −Kinetin | +Kinetin | Increment |
| | *μmoles O₂/g fresh wt·hr* | | |
| Control | 8.92 ± 0.08[1] | 9.21 ± 0.00 | 0.29 |
| KCN, 1 mM | 3.49 ± 0.06 | 3.62 ± 0.09 | 0.13 |
| Control | 3.57 ± 0.20 | 3.93 ± 0.02 | 0.36 |
| Fluoroacetate, 4 mM | 2.87 ± 0.02 | 3.02 ± 0.02 | 0.15 |
| Control | 4.46 ± 0.09 | 4.86 ± 0.15 | 0.40 |
| Iodoacetate, 5 μM | 2.70 ± 0.10 | 3.00 ± 0.17 | 0.30 |
| Control | 4.84 ± 0.03 | 5.23 ± 0.11 | 0.39 |
| NaF, 0.5 mM | 3.62 ± 0.10 | 3.76 ± 0.06 | 0.14 |
| Control | 4.94 ± 0.06 | 5.50 ± 0.07 | 0.56 |
| 2,4-DNP, 5 μM | 8.10 ± 0.09 | 8.65 ± 0.06 | 0.55 |

[1] Standard deviation in this and all subsequent tables.

Table II. *Effect of Increasing Iodoacetate Concentrations on the Respiration of Soybean Tissue and on the Kinetin Promotion Increment*

| Treatment | Rate of Respiration | | |
|---|---|---|---|
| | −Kinetin | +Kinetin | Increment |
| | *μmoles O₂/g fresh wt·hr* | | |
| Control | 3.41 ± 0.02 | 3.73 ± 0.05 | 0.32 |
| Iodoacetate, 5 μM | 3.14 ± 0.05 | 3.34 ± 0.02 | 0.20 |
| Control | 5.09 ± 0.04 | 5.68 ± 0.02 | 0.59 |
| Iodoacetate, 7.5 μM | 2.88 ± 0.07 | 2.99 ± 0.08 | 0.11 |
| Control | 4.81 ± 0.07 | 5.13 ± 0.05 | 0.32 |
| Iodoacetate, 10 μM | 1.60 ± 0.04 | 1.56 ± 0.02 | −0.04 |

Table III. *Effect of Organic Acids on Respiration of Soybean Tissue with or without 1 μM Kinetin and on the Kinetin Promotion Increment*

All substrates were tested in mannitol medium.

| Treatment, pH | Rate of Respiration | | |
|---|---|---|---|
| | −Kinetin | +Kinetin | Increment |
| | *μmoles O₂/g fresh wt·hr* | | |
| Control, 5.8 | 7.67 ± 0.17 | 8.49 ± 0.09 | 0.82 |
| Malate, 3 mM, 5.8 | 8.14 ± 0.06 | 8.77 ± 0.02 | 0.63 |
| Control, 5.8 | 7.24 ± 0.05 | 7.69 ± 0.09 | 0.45 |
| Succinate, 1 mM, 5.8 | 7.58 ± 0.00 | 7.88 ± 0.08 | 0.30 |
| Succinate, 3 mM, 5.8 | 7.90 ± 0.06 | 7.81 ± 0.02 | −0.09 |
| Control, 5.0 | 7.12 ± 0.09 | 7.67 ± 0.05 | 0.55 |
| Acetate, 0.5 mM, 5.0 | 7.63 ± 0.07 | 7.80 ± 0.02 | 0.17 |
| Control, 5.8 | 9.03 ± 0.10 | 9.86 ± 0.13 | 0.83 |
| Pyruvate, 10 mM, 5.8 | 9.43 ± 0.08 | 10.21 ± 0.09 | 0.78 |
| Control, 5.0 | 6.37 ± 0.06 | 6.75 ± 0.04 | 0.38 |
| Pyruvate, 10 mM, 5.0 | 7.55 ± 0.04 | 7.94 ± 0.05 | 0.39 |

Reducing the pH to 5.0, thereby supposedly increasing uptake of pyruvate, increased respiration even more but still did not affect the increment. Table IV shows results of tests with fluoride and iodoacetate alone or in combination with 10 mM pyruvate at pH 5.0. These data appear to show that pyruvate can overcome inhibition of the KPI in the case of both of these inhibitors. Total recovery of the increment was not obtained in the iodoacetate experiment, but the fluoride results showed an almost complete recovery of the increment as well as of total respiration.

**Cytokinin Inhibitory Effects.** A few investigations have been made concerning the inhibitory effects of kinetin on respiration. Table V shows that while 200 μM adenine had no effect on respiration of tissue without kinetin it promoted respiration in the 40 μM kinetin-treated tissue about 20% and changed the increment from negative to positive. Since this could relate to effects on oxidative phosphorylation, 2,4-DNP was used to test possible high kinetin effects on this system (Table VI). 2,4-DNP at 5 μM reduced the inhibition increment about 21% while 10 μM reduced it about 34%. In further tests, the remainder of the KPI was insensitive to additional 2,4-DNP. Similar results were obtained with CCCP.

Table IV. *Inhibition by NaF and Iodoacetate of the Kinetin Promotion Increment and Recovery from the Inhibition with Pyruvate*

| Treatment | Rate of Respiration | | |
|---|---|---|---|
| | −Kinetin | +1 μM Kinetin | Increment |
| | *μmoles O₂/g fresh wt·hr* | | |
| Control, pH 5 | 5.37 ± 0.03 | 5.91 ± 0.03 | 0.54 |
| NaF, 1 mM | 4.85 ± 0.01 | 4.93 ± 0.04 | 0.08 |
| NaF, 1 mM + 10 mM pyruvate | 5.21 ± 0.03 | 5.69 ± 0.06 | 0.48 |
| Control, pH 5 | 8.19 ± 0.03 | 8.69 ± 0.04 | 0.50 |
| Iodoacetate, 10 μM | 6.65 ± 0.04 | 6.95 ± 0.02 | 0.30 |
| Iodoacetate, 10 μM + 10 μM pyruvate | 7.20 ± 0.04 | 7.61 ± 0.03 | 0.41 |

Table V. *Adenine Reversal of the Inhibition of Respiration by Kinetin*

| Treatment | Rate of Respiration | | |
|---|---|---|---|
| | −Kinetin | +40 μM Kinetin | Increment |
| | *μmoles O₂/g fresh wt·hr* | | |
| Control | 7.29 ± 0.03 | 6.20 ± 0.07 | −1.09 |
| Adenine, 200 μM | 7.33 ± 0.05 | 7.56 ± 0.04 | 0.23 |

Table VI. *Effects of 2,4-Dinitrophenol on the Kinetin Inhibition of Respiration at pH 5.8*

| Treatment | Rate of Respiration | | |
|---|---|---|---|
| | −Kinetin | +40 μM Kinetin | Increment |
| | *μmoles O₂/g fresh wt·hr* | | |
| Control | 8.12 ± 0.09 | 6.74 ± 0.09 | −1.38 |
| 2,4-DNP, 5 μM | 10.19 ± 0.02 | 9.10 ± 0.16 | −1.09 |
| 2,4-DNP, 10 μM | 11.20 ± 0.10 | 10.29 ± 0.02 | −0.91 |

## DISCUSSION

Since adenine did not affect respiration in the concentration range tested, the kinetin and zeatin promotions seem to be specific cytokinin effects and were not due to adenine as an impurity or gross breakdown product of the hormones. In addition, the effects of various cytokinin concentrations on oxygen uptake mirror results obtained on overall growth in this same tissue (5). The optimum concentration of kinetin for both growth and respiration lies near 5 $\mu$M and higher concentrations lead to inhibition. The inhibition with high kinetin concentrations and its absence with equivalent concentrations of zeatin further supports this being a specific cytokinin effect. For example, relatively high concentrations of kinetin sharply inhibit fresh weight increases of tobacco callus while equimolar concentrations of zeatin do not (10). Since the kinetin promotion and inhibition are probably due to different effects they are discussed separately below.

The relationship between the initial kinetin action and its promotion of respiration cannot be definitely ascertained from our data. However, the 2- to 4-hr lag before expression of the KPI, the inhibition preceding it, and the small promotion finally obtained, suggest the effect is not direct. The possibility of kinetin effects on respiration through action on phosphate (4,12), potassium (8), and sugar (1) uptake was ruled out since their omission from the medium did not reduce the KPI. The time course of respiratory rate changes in the soybean tissue is quite similar to the pattern seen with sliced and aging tissue and therefore may result from changes in microsomal respiration (9). If so, the cytokinin may in some way be influencing the increase of respiratory activity in cell parts represented by the microsomal fraction. Investigators particularly interested in wounding and aging effects may find the soybean tissue quite useful.

Inhibitor studies showed that at least 90% of the respiration of this tissue is contributed by the glycolysis-tricarboxylic acid cycle pathway (unpublished results) so investigations of the KPI centered on these pathways. This respiration is largely under the control of ATP levels since 2,4-DNP effects a 60% increase in respiratory activity. Hence, any cytokinin promotion of growth or of any other energy requiring process going on during the oxygen monitoring period should result in increased respiration. An increase achieved in this way should be largely eliminated by the use of uncouplers of oxidative phosphorylation. Since no effect of 2,4-DNP (or CCCP) on the KPI was found, it follows that the kinetin action likely is not directly on ATP levels or on oxidative phosphorylation during the monitoring period. This, of course, does not rule out the possibility that oxidative phosphorylation is required for cytokinin-induced changes which occur prior to monitoring and which give the cell an increased capacity for oxygen consumption.

The sensitivity of the KPI to fluoroacetate indicates involvement of the tricarboxylic acid cycle while the effects of iodoacetate and fluoride suggest a role for glycolysis. Since malate, succinate, and acetate all decrease the KPI by promoting control tissue respiration more than that of kinetin-treated tissue, kinetin appears to be increasing substrate entering the tricarboxylic acid cycle rather than acting directly on some other aspect of the cycle. The absence of a pyruvate effect on the KPI, even though pyruvate reverses the lowering of the KPI by inhibitors of glycolysis, suggests that a point of control is related to some utilization of pyruvate. Several possibilities exist for investigation. The fact that malonate can give a cytokinin-like effect in isoflavone synthesis (6) should not be overlooked. Interpretation at this point should be of a cautious nature, however, since in the present study the inhibitors and substrates were present for extended periods (all except 2,4-DNP) and they might be affecting development of an increased capacity for $O_2$ consumption rather than influencing the uptake directly.

The kinetin inhibition of respiration appears to be a complex phenomenon, a fact mirrored by the number of explanations in the literature (3, 11). The 2,4-DNP results presented here suggest that high kinetin levels do affect oxidative phosphorylation to some extent; however, two-thirds of the inhibition cannot be explained in this manner. A final explanation of the kinetin inhibition must be capable of accounting for the complete reversal of inhibition by adenine.

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