Comparative Effects of Ethylene and Cyanide on Respiration, Polysome Prevalence, and Gene Expression in Carrot Roots

MARK L. TUCKER and GEORGE G. LATIES
Department of Biology and Molecular Biology Institute, University of California, Los Angeles, California 90024

ABSTRACT

Treatment of carrot roots (Daucus carota L.) with 10 microliters per liter ethylene in O₂ evokes a three- to four-fold increase in polysome prevalence and associated poly(A)⁺ RNA. The increase in polysome prevalence is attended by a similar change in CO₂ evolution. The increase in polysomal poly(A)⁺ mRNA constitutes primarily a generic increase in constitutive mRNAs as assayed by in vitro translation. However, changes in the relative abundance of several in vitro translatable ethylene specific mRNAs do occur.

Cyanide, at concentrations which inhibit cytochrome oxidase, initiates a respiratory rise very similar in kinetics and magnitude to that evoked by ethylene. Moreover, the combined treatment with cyanide and ethylene evokes a respiratory response resembling that caused by ethylene or cyanide alone. Nevertheless, cyanide, in the presence of ethylene, significantly inhibits the increase in polysome prevalence and new gene expression associated with ethylene treatment of carrot roots. Separation of in vitro translation products by one-dimensional and two-dimensional gel electrophoresis shows that several new in vitro translation products appear in cyanide-treated carrots different from those evoked by ethylene.

Engagement of the less energy efficient alternative electron transport path by cyanide may be responsible for inhibition of the normal ethylene associated increase in polysome prevalence and new gene expression. The implications of these results on regulation of respiratory metabolism are discussed and compared with the results for similar experiments with avocado fruit (Tucker and Laties 1984 Plant Physiol 74: 307-315) in which cyanide does not inhibit an ethylene induced increase in polysome prevalence and change in gene expression.

Ethylene, a natural plant hormone, induces an increase in the respiration of many fruits and bulky plant storage organs (18-20). Solomos and Laties (20) showed that in several plant tissues cyanide, at concentrations that inhibit Cyt oxidase, causes an increase in respiration very similar in kinetics and magnitude to that induced by ethylene. In avocado fruit, cyanide evokes a normal ripening response akin to that caused by ethylene, as determined by changes in respiration, polysome prevalence, and gene expression (24).

Cyanide, in addition to inhibiting Cyt oxidase, may act as an ethylene analog, binding to an ethylene receptor site. Cyanide has several chemical characteristics of an ethylene analog (16), and at 2,000 μl/l inhibits ethylene binding in a mung bean in vitro assay for ethylene binding sites (17). However, it is not possible to establish unequivocally in avocado fruit that cyanide is acting in the capacity of an ethylene analog because cyanide initiates ethylene production in avocado (24).

Christoffersen and Laties (4) showed that in carrot roots ethylene, in addition to causing an increase in respiration, induces an increase in polysome prevalence and a change in gene expression when roots are exposed for 24 h to 10 μl/l ethylene in air or O₂, the response to ethylene being greater in O₂. Inasmuch as carrot roots treated with 500 μl/l cyanide in O₂ do not synthesize detectable levels of ethylene, it is possible to separate and compare the cyanide and ethylene response in carrot roots.

Plant tissues that show an increase in respiratory rate upon exposure to cyanide possess a cyanide-insensitive, alternative respiratory path (20). In connection with an early proposal that the alternative path mediates the respiratory climacteric (20) it was suggested that ethylene may favor the operation of the alternative path at the expense of the Cyt path. Nevertheless, no effect of ethylene on the Cyt path of isolated mitochondria was demonstrable (1). Furthermore, experiments involving inhibitor studies of 1 mm fresh slices taken from ethylene-treated intact avocado and banana fruit invariably demonstrate that the alternative path is not used by slices taken from ethylene-treated fruit (23). However, the rate of respiration of fresh slices is consistently different from that of the parent organ (23). It is possible, therefore, that the nature and/or regulation of respiratory metabolism in 1 mm slices differs from that in the intact organ.

The alternative, cyanide-insensitive electron transport path makes a single ATP when linked to the oxidation of mitochondrial NADH, whereas the Cyt path makes three (25). Tucker and Laties (24) concluded that in avocado fruit ripening, where cyanide does not inhibit ethylene educed changes in polysome prevalence and gene expression, the less energy efficient alternative path is capable of fulfilling the energy requirements of these processes.

In what follows, in order better to understand the interrelationship of respiration, polysome prevalence, and gene expression in ethylene-treated carrot roots, and to compare same with the changes that occur in response to cyanide, the temporal course of these events in each case was followed from 0 to 24 h after treatment. In carrot roots it is shown herein that cyanide does not affect the respiratory climacteric evoked by ethylene but does inhibit the increase in polysome prevalence and change in gene expression associated with ethylene treatment—presumably as a result of its inhibition of oxidative phosphorylation.

MATERIALS AND METHODS

Treatment of Plant Material. Carrot roots (Daucus carota L.) were purchased locally. Within a few hours of purchase, five carrot roots weighing approximately 400 g total were placed in 4-L glass jars maintained at 25°C. Roots were swept with a
FIG. 1. Effect of 10 μl/l ethylene and/or 500 μl/l cyanide in O2 on (A) CO2 evolution, (B) polysome prevalence, and (C) polynuclear poly(A)* RNA in carrot roots. Polysome prevalence calculations were adjusted to exclude RNA associated with monosomes and ribosomal subunits which were partially sedimented along with polysomes (see Fig. 2). Poly(A)* RNA determinations included poly(A)* RNA associated with all fractions sedimenting with polysomes, including monosomes (see Fig. 2). (×), untreated; (○), ethylene; (△), cyanide; (□), ethylene and cyanide. Each point in (C) is the average of 2 to 6 samples. The bars in (C) indicate ± of the mean. Lines connecting points at 0 and 24 h in cyanide and ethylene treated roots in (B) and (C) are simply for clarity, and do not represent the kinetics of the observed concentration changes through 24 h.

continuous 50-ml/min flow of water-saturated O2 for 24 h prior to treatment with 10 μl/l ethylene and/or 500 μl/l cyanide in O2. Ethylene at 10 μl/l in air was administered as a mixture of compressed gases. Cyanide was added to the gas stream by bubbling O2 or ethylene and O2 through 500 ml of buffered solution (Tes 20 mm, pH 7.0) having an initial concentration of KCN of 5 mM—which is in equilibrium with 500 μl/l HCN in the gas phase at 25°C [11]. CO2 production was monitored automatically every hour by passing the effluent gas through an IR gas analyzer (Anarak, model AR500).

Cyanide Measurement. Cyanide in the gas stream was measured by bubbling 25 ml of a gas sample through 8 ml of indicator solution (39 μM phenolphthalein, 0.1 mM CuSO4, 4 μM Na2HPO4, adding 2 ml 20 mM K2HPO4, and measuring the ensuing color spectrophotometrically at 552 nm [12]). Cyanide in the tissue was measured as follows: carrot roots were sliced into liquid N2 immediately after removal from the respiration jar. Five g of frozen tissue was homogenized at 4°C in 45 ml of 50 mM NaOH and 100 mg/l sodium 2-mercaptoethanolase as a polyphenol oxidase inhibitor. In an evacuated closed system, 2 ml of 1 M lactic acid was added to 5 ml homogenate, and the mixture heated to distill cyanide into 8 ml of the designated indicator solution. After distillation of cyanide was complete, 2 ml of 20 mM KOH was added and the resulting color quantified spectrophotometrically at 552 nm [12].

FIG. 2. Effect of 10 μl/l ethylene in O2 on polysome profiles obtained from carrot roots. Each profile represents 1.4 g of carrot tissue. Peaks to the left of dashed lines were excluded in calculations of polysome prevalence (Fig. 1B), but any poly(A)* RNA associated with these peaks was included in quantification of poly(A)* RNA as well as in in vitro translations (Figs. 1C, 3, and 5).

Polysome Preparation and Polysomal Poly(A)* RNA Isolation. Two carrot roots from each sample were randomly selected and a 15-g cross-section cut from the middle of each carrot. The two sections weighing 30 g total were homogenized together in an Oster vegetable juicer with 100 ml of cold (4°C) polysome extraction buffer (0.5 M sucrose, 200 mM Tris-HCl, pH 9.0, 400 mM KCl, 35 mM MgCl2, 25 mM EGTA, 5 mM 2-mercaptoethanol). The coarse debris was removed by filtration of the homogenate during juicing through a strip of Miracloth lining the basket centrifuge of the juicer. Further isolation and purification of polysomes and associated poly(A)* RNA was accomplished essentially as described by Christoffersen and Laties [4]. The procedure for poly(A)* RNA isolation, however, entailed two additional steps to remove contaminating polysaccharides from the ethanol precipitate of extracted polysomal RNA. The ethanol precipitate was suspended in 3.6 ml of elution buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA; 0.1% SDS). The suspension was heated to 80°C for 3 min, rapidly cooled to room temperature, and 0.4 ml 5 M NaCl added. The suspension was then centrifuged at 10,000 g for 5 min producing a pellet which was discarded. To the supernatant was added 2.5 volumes of cold (-20°C) ethanol to precipitate RNA. The RNA precipitate was suspended again in elution buffer, heated to 80°C for 3 min, cooled, 0.1 volume of 5 M NaCl added, and the suspension passed through a cellulose column equilibrated with 0.5 mM NaCl in elution buffer (8). The eluate was then passed through an oligo dt-cellulose affinity column, and poly(A)* RNA subsequently released with elution buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA; 0.1% SDS). To ensure the purity of the poly(A)* RNA fraction, 0.1 volume of 5 M NaCl was added to the eluate and it was passed a second time through the oligo dt-cellulose column and poly(A)* RNA subsequently released with elution buffer.

In Vitro Translation. A wheat germ in vitro translation extract was prepared as described by Roberts and Paterson [13]. The extract was not nuclease treated as has become traditional due to nuclease treatment inhibited [35S]methionine incorporation in all samples. The ratio of [35S]methionine incorporation with 0.5 μg exogenous mRNA to that with no exogenous RNA...
two-dimensional SDS electrophoresis, the mixture was incubated lacking methionine, 50 mM GTP, 20 mM phosphocreatine, 50 μg/ml creatine phosphokinase, 2 mM DTT, 30 μM each of a combination of amino acids lacking methionine, 60 μg placental ribonuclease inhibitor (Bio- tec), and 15 μCi (5.6 × 10⁵ Bq) [³⁵S]methionine (>600 Ci/mmol, Ameraham). Translation was started upon bringing the volume to 50 μl with 0.5 μg poly(A)⁺ mRNA in water. Samples were incubated for 1 h at 25°C. The reaction was stopped on ice and the mixture stored at −20°C.

Electrophoresis of In Vitro Translation Products. For one-dimensional electrophoresis, aliquots of translation products (approximately 2 μl/sample) containing 2 × 10⁵ cpm TCA precipitable [³⁵S]methionine were suspended and boiled 3 min in 25 μl of SDS sample buffer (63 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), and electrophoresed on a 10 to 15% polyacrylamide gradient gel containing 1% SDS (6). For two-dimensional electrophoresis, an aliquot containing 2 × 10⁵ cpm of TCA precipitable [³⁵S]methionine was suspended in 20 μl of 9 M urea, 4% Nonidet NP40, 2% ampholytes, and 2% 2-mercaptoethanol. Samples were electrophoresed essentially as described by O’Farrell (9). Buffers used for isoelectric focusing in the first dimension of two-dimensional gels were 10 mM iminodiacetic acid in the anode chamber and 10 mM ethylenediamine in the cathode chamber. Two-dimensional gels were electrophoresed in the second dimension in a 10 to 15% polyacrylamide gradient gel containing 1% SDS (6). Both one- and two-dimensional gels were fixed, stained with Coomassie blue R-250 to identify molecular weight markers, infiltrated with 2,5-diphenyloxazole, dried, and exposed to Kodak XAR-5 X-ray film (7). One-dimensional fluorographs were scanned for quantification using a Joyce, Loeb & Co. Ltd. microdensitometer.

RESULTS

Ethylene Response. Carrot roots treated with 10 μl/l ethylene in O₂ show a 3- to 4-fold increase in polysomes and associated polysomal poly(A)⁺ RNA (Figs. 1 and 2). The increase in polysome prevalence and associated poly(A)⁺ RNA coincides with or slightly precedes the increase in CO₂ evolution (Fig. 1). The seemingly more rapid increase of poly(A)⁺ RNA in Figure 1C is not considered significantly different from the rate of increase of total polysomal RNA in Figure 1B.

The centrifugation procedure used for sedimentation of polysomes does not fully sediment monosomes and ribosomal subunits—the first three peaks in each profile shown in Figure 2. However, polysomes are nearly 100% sedimented (Richard Boyd, personal communication). Since a variable fraction of the monosomes and ribosomal subunits is sedimented, it is not possible to draw conclusions concerning the relative ratios of these components to each other. For the same reason, attempts to relate the drop in monosomes and subunits to the increase in polysomes upon treatment with ethylene are not justified, and estimation of polysome prevalence excludes the monosome and subunit peaks shown in Figure 2.

As determined by in vitro translation of poly(A)⁺ RNA, several changes in gene expression occur in response to ethylene treatment (Figs. 3 and 5). The appearance and accumulation of several ethylene specific translatable mRNAs occur at approximately the same time that total polysomal poly(A)⁺ RNA begins to increase (compare Figs. 1C and 4). Only the most pronounced and repeatable changes in translation products are designated with symbols in Figure 3 and quantified for plotting in Figure 4.
Fig. 5. Fluorograph of two-dimensional (2-D) and one-dimensional (1-D) gel electrophoresis of in vitro translation products of polysomal poly(A)+ RNA extracted from (A) untreated (0 h) and (B) 24 h ethylene treated carrot roots. Symbols to the right of the 1-D fluorographs refer to specific mol wt in vitro translation products which change with ethylene treatment as shown in Figure 3. Boxes and circles within 2-D fluorographs enclose translation products which appear to increase and decrease respectively with ethylene treatment of carrot roots.
25°C is in equilibrium with 500 μl/l cyanide in the gas phase (11), under the conditions of these experiments carrot roots exposed for 24 h to 500 μl/l cyanide in the gas stream reach a cyanide concentration of 1.0 ± 0.13 mM. Since the apparent Kf for cyanide inhibition of Cyt oxidase is approximately 10 μM (21–23), the cyanide concentration in carrots in these experiments is considerably higher than necessary to inhibit Cyt oxidase completely.

Although cytochrome oxidase is fully inhibited by cyanide, the respiratory climacteric evoked by cyanide is very similar in kinetics and magnitude to that evoked by ethylene (Fig. 1A). However, exposure of carrot roots to cyanide for 24 h caused but a 2-fold increase in polyosome prevalence relative to that in untreated roots, in contrast to the 3- to 4-fold increase observed in ethylene treated roots (Fig. 1B). The disparity was reaffirmed by measurements of poly(A)+ RNA in carrot roots treated for 24 h. In four separate experiments the mean value of poly(A)+ RNA per gram tissue of cyanide treated roots was about twice that of untreated roots, whereas the increase of poly(A)+ RNA above the controls in ethylene treated roots was 4-fold (Fig. 1C).

One- and two-dimensional electrophoresis of in vitro translation products of polysomal poly(A)+ RNA shows a marked change in gene expression in carrot roots exposed to cyanide for 24 h (Fig. 6; Fig. 5A compared with Fig. 7A). However, the cyanide educed change is different from that induced by ethylene alone (Fig. 6; Fig. 7A compared with Fig. 5B). Nevertheless, cyanide affects the accumulation and disappearance, although to a lesser extent, of some messages affected by ethylene, as noted in a comparison once again of Figure 7A with Figure 5B.

The Combined Effect of Cyanide and Ethylene. Exposure of carrot roots to a combination of cyanide and ethylene for 24 h caused a change in polyosome prevalence and poly(A)+ RNA more closely resembling that due to cyanide than to ethylene (Fig. 1, B and C). The respiratory climacteric, however, is very similar to that evoked by cyanide and by ethylene alone (Fig. 1A). The combination of ethylene and cyanide produces an electrophoretic pattern of in vitro translation products largely similar to that elicited by cyanide alone (Fig. 7, A and B). The addition of ethylene may, however, enhance both the accumulation and disappearance of ethylene induced messages (Fig. 7B).

**DISCUSSION**

Christoffersen and Laties (4) reported that in carrot roots the magnitude of the ethylene evoked increase in polysomes after 24 h exposure to ethylene in air or O2 correlated with the respective enhancement of respiration, the magnitude of the increase being approximately 2-fold in air and 4-fold in O2. We have shown herein that treatment of carrot roots with ethylene in O2 evokes an increase in polysomes within 3 h which continues throughout 24 h, without further change through 24 h. The ethylene evoked increase in polysomes and associated poly(A)+ RNA is accompanied by an increase in CO2 evolution. These observations suggest that ethylene causes an increase in protein synthesis, and thereby elicits a respiratory increase in response to the energy demand. This interpretation is supported by Chalutz's finding (2) that carrot roots treated with ethylene in air for 48 h showed a 50% increase in protein content. Alternatively, the rise in respiration in response to ethylene may be a characteristic of ethylene action independent of energy demand (24).

In connection with the evocation by ethylene in both carrot and avocado (24) of an increase in polysomes and associated poly(A)+ RNA, as well as a change in gene expression, in both cases the bulk of the increase in polysomal poly(A)+ RNA represents a generic increase in constitutive mRNA (cf [15]). The ethylene evoked generic increase in poly(A)+ mRNA may be the result of a change in transcriptional and/or posttranscriptional...
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Fig. 7. Fluorograph of two-dimensional gel electrophoresis of in vitro translation products of polysomal poly(A)+ RNA extracted from 24 h (A) cyanide and (B) cyanide plus ethylene treated carrot roots. Boxes and circles within fluorographs are the same as in Figure 5 and refer to translation products which increase or decrease, respectively, with ethylene treatment of carrot roots. Several polypeptides appear to increase with respect to cyanide treatment; however, these are not enclosed to avoid figure complexity.

control, and may well be a common, general response to ethylene.

An increase in ribosome packing on message attending the polysome increase was previously reported (4). The evidence for this was that after 24 h of exposure to ethylene there was a doubling of the ratio of total polysomal RNA to poly(A)+ RNA isolated from polysomes, as well as an approximate doubling from pentamer to decamer of the average polysome size as determined by rate zonal centrifugation of polysomes in a sucrose gradient. In the present study, neither the average size of polysomes in polysome profiles in Figure 2 nor the ratio of total polysomal RNA to poly(A)+ RNA in Figure 1 changed signifi-
cantly with 24 h of ethylene treatment. A more definitive study of packaging might be obtained from electron microscopy studies of polysomes from ethylene treated and untreated tissue.

The treatment with cyanide and ethylene together, as well as with cyanide alone, evokes an increase in respiration very similar to that caused by ethylene. However, the combination results in a much reduced increase in polysome prevalence and polysomal poly(A)^+ RNA (Fig. 1). Since cyanide diverts electron transport through the less energy efficient alternative path (18), this may be reason enough for the inhibition of the ethylene associated increase in polysome prevalence. Why, however, should the kinetics and magnitude of the respiratory climacteric elicited by ethylene and by cyanide, or ethylene plus cyanide be so similar?

As noted, the ethylene response may reflect a spate of biosynthesis, with attendant respiration stimulation in response to energy demand. The gross respiratory response to cyanide in turn may be comprehensible in terms of a pseudouncoupling phenomenon that is responsible for a substrate-mobilizing Pasteur effect. It is to be expected, however, that operational uncoupling would lead to a prompt respiratory rise, yet the kinetics of the development of the induced respiration is the same in ethylene as in cyanide, i.e., drawn out and sigmoid. Parenthetically, were the kinetics of the developed rate reflective of gas penetration—ethylene or cyanide—the expected time-course would be hyperbolic.

Accordingly, cyanide must be viewed as having several effects. On the one hand, an array of new messages, distinct from those induced by ethylene, are induced by cyanide—as evidenced by the collection of cyanide-specific translation products noted in the central region of the gels shown in Figure 7, A and B. Whether the messages are induced by cyanide per se, or by some product of cyanide-altered metabolism, is problematical. It is noteworthy in this connection that, for whatever reasons, the respiratory climacteric in potato tubers is evoked by vapors of cyanide, ethanol, acetaldehyde, or acetate, much as by ethylene—with respect to time-course, maximal rate, and synergistic response to O_2 (3, 5, 14). The kinetics and magnitude of the respiratory climacteric in carrot roots can only secondarily, if at all, be due to the biosynthesis of specific proteins, since cyanide alone evokes a different complement of mRNAs than does ethylene (Fig. 6; and Fig. 5 compared with Fig. 7). Second, as noted, cyanide causes a respiratory climacteric similar in magnitude and time-course to that caused by ethylene. In this connection cyanide acts as an ethylene-like elicitor. Finally, cyanide inhibits Cyt oxidase and diverts electron flow through the alternative path, thus bringing about a degree of operational uncoupling that diminishes protein synthesis as reflected in the lowered level of polysome formation.

Although cyanide may act as a competitive inhibitor of ethylene (16, 17), and thus, perhaps, prevent message induction by ethylene (e.g., compare the central rectangle in Fig. 5B with that in Fig. 7B), cyanide does not affect ethylene-induced gene expression in avocado (24), and it is therefore more likely that cyanide inhibits ethylene induced gene expression in carrots through its effect on energy metabolism. In avocado fruit ripening the alternative path sustains seemingly all of the associated changes in polysome prevalence and gene expression (24), whereas in carrot much, though not all, of what transpires in response to ethylene depends on a fully active cytochrome path. That is, the phosphorylation level in the presence of cyanide is adequate to sustain ethylene induced protein synthesis in avocado, but not in carrot.

**LITERATURE CITED**

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