Respiration and Embryogenesis in Cotton[1, 2]

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Introduction

Virtually nothing is known about the metabolism associated with embryogenesis in higher plants, not even the oxidative pathways involved in respiration. This contrasts sharply with the situation in the field of animal embryology where a large amount of data dealing with the biochemistry of embryo development has been collected (3). When embryogenesis is defined as the result of maintenance, growth, and differentiation (3), it becomes increasingly apparent that it is important to understand the basic respiratory pathways operating in embryos and to examine the possibility of pathway changes during the course of development.

The experiments described below are concerned with the relation of respiration to development of the plant embryo. Three areas investigated were 1) the normal respiration of the plant embryo, 2) the characterization of the oxidative pathway involved in respiration through the use of respiratory enzyme inhibitors, and 3) the histochemical localization of relative respiratory activities during the early stages of embryogenesis.

Materials and Methods

The embryos used in this study were from Upland Cotton, *Gossypium hirsutum L.* seed variety M8948, which is a double haploid developed by Dr. James Meyers of the Delta Branch Experimental Station, Stoneville, Mississippi. The plants were grown in a green house with a minimum temperature of 30°. Fluorescent lighting extended the day length to 18 hours. Cotton grown under these conditions flowers all year. When the cotton flower opens, the stigma is receptive to pollen for 1 day. The flowers were hand pollinated and all stamens were removed. Fertilization occurs approximately 18 hours following pollination. The embryos were allowed to develop on the plant with the individual bolls selected as needed.

Oxygen Uptake. Since only a limited number of embryos can be removed from a boll within a practical period of time, it was necessary to employ the Cartesian diver microgasometer (7, 10) for the quantitative portions of this work. The divers used had a neck diameter of 1.0 mm and a volume of 10 μl.The divers were filled in the following manner. The lowermost charge, placed in the bulb of the diver, consisted of a 0.6 μl drop of 0.1 M NaOH. The tissue seal consisted of a 0.5 μl drop of 0.06 M $NaH_2PO_4-K_2HPO_4$ buffer at pH 6.8. This was followed by the standard paraffin oil seal of 0.6 μl. The final charge was the mouth seal for which the volume was calculated from the length of the seal when the diver was at equilibrium in the flotation vessel.

Respiration, as measured by O_2 uptake, was determined for the early developmental stages of the embryo. Embryos in these stages of development were excised from intact bolls. Following excision the embryos were immediately washed in 3 changes of the phosphate buffer solution to remove any residual endosperm. The amount of endosperm adhering to the external surface of the embryo following the washes was negligible. The isolated embryo was transferred with a braking pipette to minimize the possibilities of injury. Before placing the embryo into the diver, a silhouette of the embryo profile was photographed. The embryo was then placed directly into the tissue seal of the diver. This was accomplished by completely filling the diver neck with the tissue seal solution, placing the embryo on top, and allowing it to fall to the bottom meniscus by gravitational force. The excess liquid was removed and the exact volume of the tissue seal determined with the aid of a horizontal microscope equipped with a calibrated ocular micrometer. The remaining seals were added, and the diver was introduced into the flotation vessel. After an initial period of equilibration, measurements were begun. All experiments were carried out at 30°. In the experimental system employed, 7 flotation vessels could be used at 1 time. A maximum of 6 experimental divers and 1 control diver, filled in an identical manner but without an embryo, were run simultaneously. The range of developmental stages (fig 1 a–l) for which O_2 uptake data was obtained included globular embryos (75–100 μ in length) through torpedo stage embryos (750 μ in length).

Inhibition of Respiration. Fluoroacetic acid and malonic acid were used to investigate the respiratory pathways operating in the developing embryo. For the inhibition studies use was made of the side drop technique (9) employed with the Cartesian diver. In the case of fluoroacetate a single side drop, 0.3 μl in volume with a concentration of 0.026 M, was used (final concentration after mixing with tissue seal 0.01 M). Two side drops were employed for

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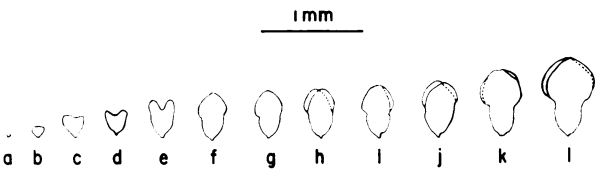

FIG. 1. Outline drawings of embryos made from photographs of the developmental stages included in study [globular stage (a) through torpedo stage (1)].

the malonate study. One side drop, nearest the tissue seal, contained 0.3 μl of 0.026 M sodium malonate (final concentration of the malonate was 0.01 M following mixture with the tissue seal). The second side drop was 0.3 μl of 0.36 M sodium succinate (final concentration of the succinate following mixture with the malonate-tissue seal was 0.1 M). The embryonic stages studied and the handling techniques were the same as described for the O_2 uptake investigation. Inhibitory side drops were introduced after a period of measurement of normal respiration (minimum 60 min). The side drops (fluoroacetate or malonate) were mixed with the tissue seal by placing an overpressure on the diver (9). After a short period of equilibration (10–20 minutes) readings were resumed. In the case of malonate inhibition experiments, the succinate side drop was mixed in the same manner following at least a 60-minute measurement of the malonate effect.

Localization of Respiratory Activity. In addition to the quantitative measurements of respiration, a study of the distribution of respiratory activity in the developing embryo was made. Nitro blue tetrazolium has been shown to be a sensitive indicator for succinic dehydrogenase activity (11). This is apparently true at the level of the electron microscope (14) and, with proper precautions and adequate controls, at the level of the light microscope (1, 5, 6). For microscopic localizations of enzyme activity whole embryos, corresponding to the developmental stages studied in the O_2 uptake experiments, were incubated in nitro blue tetrazolium medium (Nitro-BT^r Dajac Laboratories) at 37° for 30 minutes. The incubation medium consisted of equal parts of 0.1 M sodium succinate, 0.1 M phosphate buffer pH 7.2, and 1 mg/ml aqueous nitro-BT. Controls were 1) embryos preincubated for 30 minutes in 0.01 M malonic acid followed by incubation in nitro-BT with or without additional succinate, 2) embryos in which lipid extraction had been carried out in cold acetone (2°) followed by incubation in the nitro-BT medium, and 3) embryos that had been heat inactivated. Following incubation and after squashing or cryostat sectioning (30 μ at −18°), embryo preparations were microscopically examined.

Determination of Cell Number. The growth rate of the cotton embryo varies at different times of the year. For this reason embryo area (mm^2) was used in this study as a measure of development instead of embryo age. Embryo cell numbers were determined by 1) photographing silhouettes of embryo profiles, 2) making Feulgen squashes of the embryos, 3) photographing the squashes, and 4) making counts of the number of nuclei present from photographic enlargements. As the cells are all uninucleate, this count represented the total number of cells present. The area of the embryo was determined from the silhouette photograph using an optical planimeter. It was found that embryos with the same area agreed closely in cell number and were at the same stage of morphological development. This made it possible to accurately determine the cell number of experimental embryos by simply measuring their areas (fig 2).

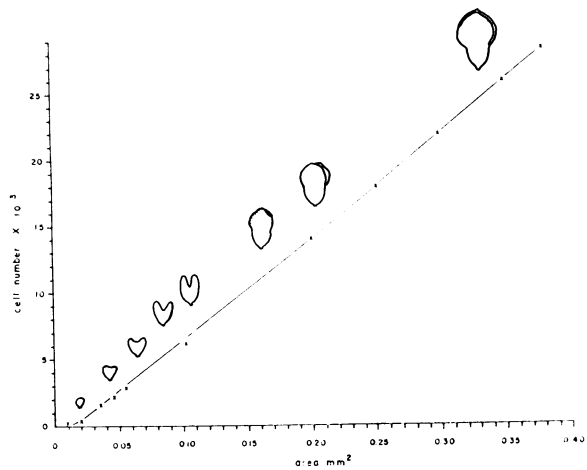

FIG. 2. The cell numbers as compared to the area of the embryos.

Results

Oxygen Uptake. The experimental data from O_2 uptake studies show that the rate and amount of O_2 consumption per embryo increases with the size and morphological development of the embryo (fig 3). In the torpedo stage the gradual decrease in the rate of O_2 consumption towards the end of the period of measurement is probably due to substrate depletion rather than O_2 limitation since the volume of the diver allows adequate amounts of O_2 to be present over the time span of the experiments.

The average amount of O_2 consumed per hour increases linearly as a function of increasing embryo size (fig 4). On a per embryo basis the O_2 consumption of globular embryos (0.007–0.02 mm^2) was about 2.5×10^{-3} μl per hour. This increased through the heart and torpedo stages with the latest torpedo stage studied (0.38 mm^2) having had an O_2 consumption of about 92×10^{-3} μl per hour. The oldest stage measured in this investigation is still a relatively immature embryo with reference to the total morphological development comprising embryogenesis.

In order to calculate the O_2 uptake on a per cell basis, use was made of the previously calculated cell numbers (fig 2). A comparison between the O_2 consumption per cell and the O_2 consumption of

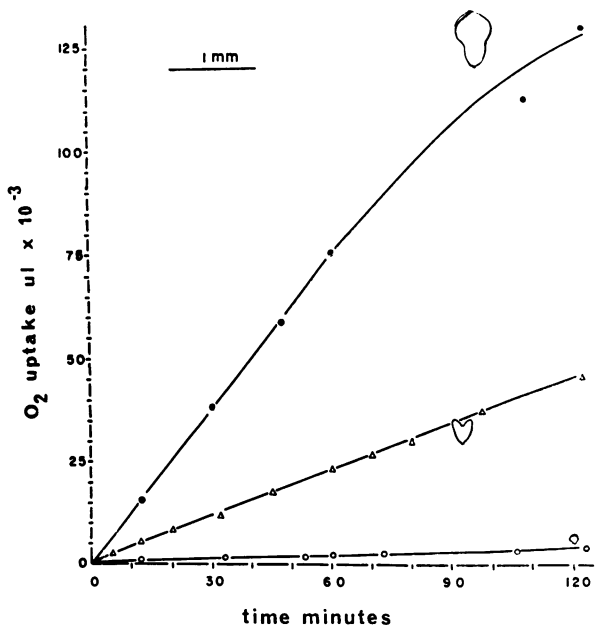

FIG. 3. O_2 uptake in the globular, heart, and torpedo stages of embryo development.

the whole embryo can be made (fig 4). Expression of this data on a per cell basis shows that the O_2 uptake is high initially (globular stage, 21.6 × 10^{-6} μl/hr), drops rapidly (middle heart stage. 6.0 × 10^{-6} μl/hr), levels off (early and middle torpedo stages, 3.4 × 10^{-6} μl/hr), and maintains this level through the later torpedo stages (3.2 × 10^{-6} μl/hr). These changes contrast markedly with the results obtained when calculated on a whole embryo basis.

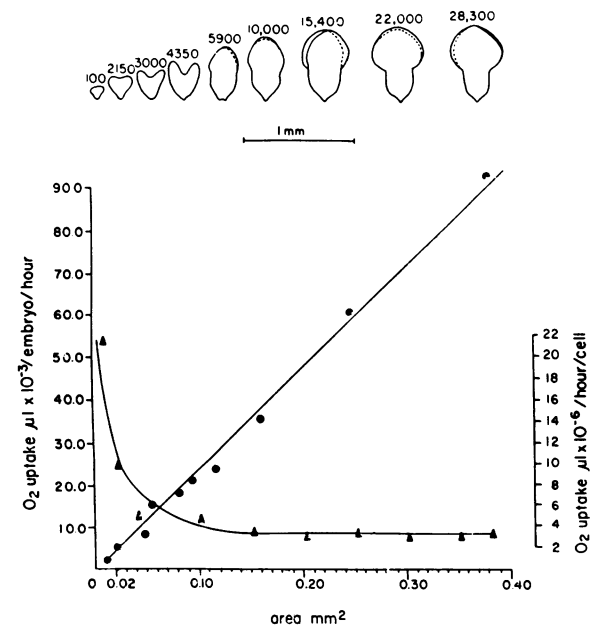

FIG. 4. Average O_2 uptake per embryo per hour (left ordinate) and average O_2 uptake per cell per hour (right ordinate). Developmental stages with cell numbers indicated are shown at the top.

Inhibition of Respiration. Fluoroacetic acid causes complete inhibition of O_2 consumption in all stages studied (fig 5). Upon the addition of the acid, O_2 uptake stops immediately in globular stage embryos. Inhibition of O_2 uptake in the heart stage is complete although it occurs more slowly than in the globular stage. Even more gradual inhibition

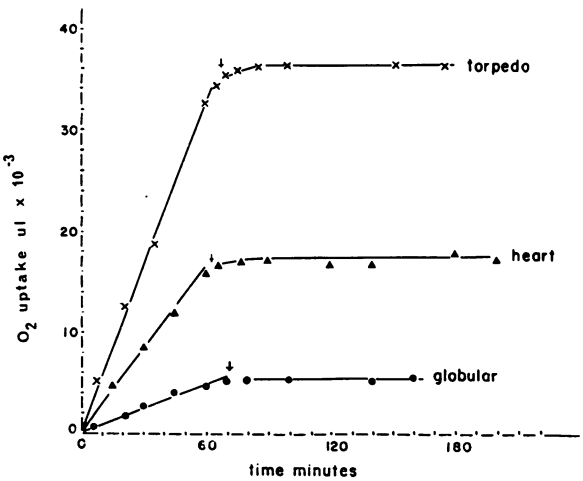

FIG. 5. Effect of fluoroacetic acid on O_2 uptake (final conc of inhibitor 0.01 M). Arrows indicate addition of the fluoroacetic acid.

was observed in the torpedo stages. The data from many embryos indicated that the more advanced the stage of development the less immediately sensitive was the embryo to fluoroacetic acid. This change in sensitivity is probably due to penetration differences associated with increasing growth and tissue complexity. Regardless of how gradual the response was, the final effect of fluoroacetic acid was to completely inhibit respiration in all stages studied.

The results of adding malonic acid (fig 6) were similar to those obtained with fluoroacetate. The globular and heart stages reacted more quickly to the inhibitor than did the torpedo stage. Again, more advanced stages were less immediately sensitive to the inhibitor. None of the embryonic stages studied showed malonic acid resistant respiration. The addition of succinate, however, caused an indication of the resumption of O_2 uptake in all stages (fig 6). This, coupled with the histochemical evidence (see below), leads to the conclusion that a reversal of the malonic acid inhibition was effected. However, more quantitative data are required.

Localization of Respiration. The distribution of respiratory activity was determined by the histochemical localization of succinic dehydrogenase activity. In the cotton embryo the localization is a particulate one corresponding to the mitochondrial distribution within the cells. This was evident in the reaction for the cells comprising the basal portion of the hypocotyl

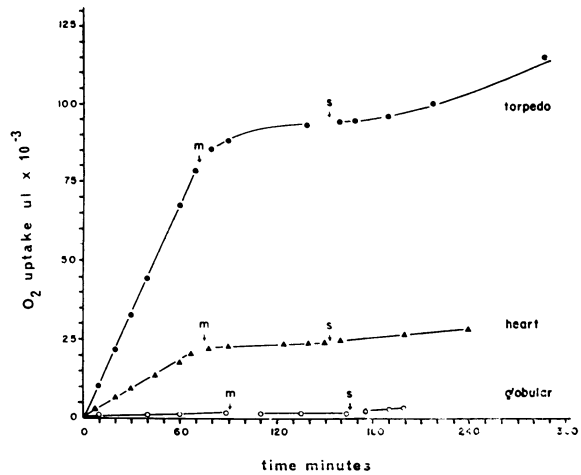

FIG. 6. Effect of malonic acid (m) followed by succinic acid (s) on O_2 uptake. Final concentration of malonate 0.01 M, final concentration of succinate 0.1 M.

(fig 7) and for the cells of the cotyledons (fig 8). In general, the intensity of the reaction in the cotyledons appeared to be less than that for the cells comprising the hypocotyl. The activity in the apex of the embryo (fig 9) was strikingly small when compared to the activities found associated with the cotyledons and subapical portions of the embryo. Control embryos pretreated with malonic acid showed no reaction for enzyme activity. The same results were ob obtained in heat inactivated embryos as in those pretreated with malonate. Lipid-extracted embryos provided a reaction pattern identical to the untreated embryos, indicating that the dinitroformazan deposition corresponded to the mitochondria and was not an artifact caused by the lipophilic properties of the dinitroformazan (6, 12). Embryos which had been inhibited by malonic acid treatment and then incubated in nitro-BT medium to which succinate had been added showed recovery as indicated by dinitroformazan deposition.

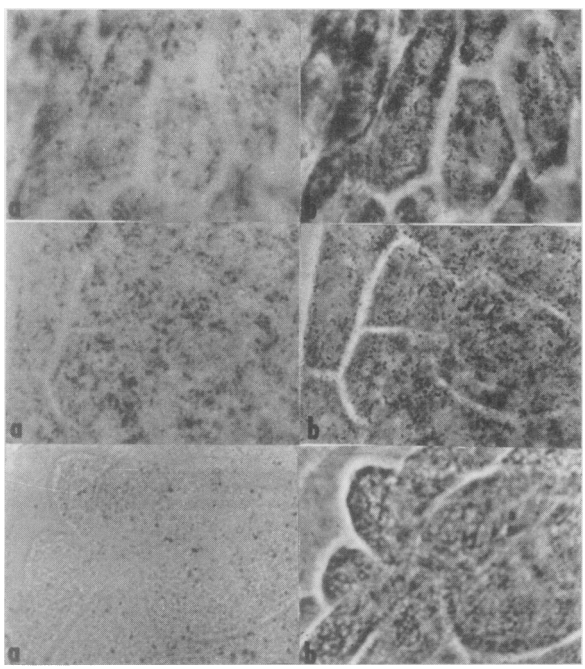

FIG. 7–9. Photomicrographs of succinic dehydrogenase localization. Nitro-BT reaction is deep purple-blue as seen with the light microscope. FIG. 7. Localization of succinic dehydrogenase activity in cells of the hypocotyl. Torpedo stage embryo: a) light microscope, b) same section under phase (X400). FIG. 8. Localization of succinic dehydrogenase activity in cells of the cotyledon. Torpedo stage embryo: a) light microscope, b) same section under phase (X400). FIG. 9. Localization of succinic dehydrogenase activity in cells of the apical region. Torpedo stage embryo: a) light microscope, b) same section under phase (X400).

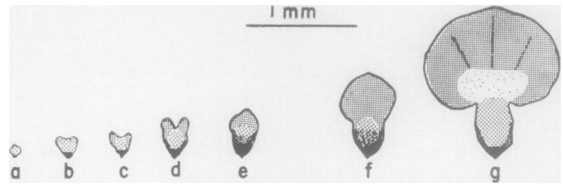

FIG. 10. A diagrammatic representation of the distribution of succinic dehydrogenase activity in the early stages of embryo development.

The distribution of enzyme activity for the early stages of embryogeny is shown in a diagrammatic representation (fig 10 a–g). Enzyme activity was evenly distributed throughout the globular stage embryo (fig 10 a) with a slightly more intense reaction in the rudimentary suspensor. In the early heart stage embryo (fig 10 b), enzyme activity was found to be associated with the developing cotyledons as well as with the basal portion of the hypocotyl. This pattern of activity continued through the middle and late heart stages (fig 10 c, d) and included peripheral extensions which eventually linked the hypocotyl with the cotyledons. At this point in development a large U-shaped pattern of enzyme activity was found. In the torpedo stages (fig 10 e, f) localization of activity was still in the cotyledonary and hypocotyl portions of the embryo while little localization was associated with the embryonic shoot apex. However, there was an increase of activity associated with the subapical portions of the embryo which corresponded to the elongating axis. A more advanced stage of development (fig 10 g), for which there is no quantitative data at present, showed an increase in enzyme activity in the developing radicle and in the longitudinal axis of the embryo. The shoot apex continued to be an area of relatively low activity. There was a decrease in activity at the base of the expanded cotyledons in this stage although enzyme activity continued to be associated with most of the cotyledonary tissue. Files of cells showing a more intense reaction occurred within the cotyledons themselves and corresponded to the developing procambium in these areas.

Discussion

O_2 uptake studies with numerous animal embryos show that the amount of O_2 consumed by the whole embryo increases linearly with size (4). The results of O_2 uptake experiments with embryos of cotton indicate a similar pattern. These data constitute the first quantitative information for the early developmental stages of a plant embryo. If O_2 consumption is expressed on a per cell basis, the pattern is strikingly different than that for the whole embryo. Instead of an increase in the amount of O_2 taken up per cell there is a decrease. Thus, the total O_2 uptake per embryo is correlated with an increase in the total number of cells comprising the embryo. Similar patterns have also been reported for animal embryos (4).

The histochemical localization of succinic dehydrogenase activity emphasizes an important aspect of the relationship between respiration and embryogeny. That is, with growth, differentiation, and increasing needs for energy, there are differences in respiratory activity within the embryo. This is demonstrated by the differential nature of the localization of enzyme activity (figs 7–9, 10 a–g). The cells of the embryo in which high enzymatic activity was localized are associated with those areas of the embryo where active growth and differentiation are taking place. This is in agreement with other studies demonstrating the close correlation of increases in respiratory activity with differentiation and active growth (2, 8, 11, 15). Cells with greater respiratory activity can also be expected to have greater O_2 uptake rates. The result is that cells with greater O_2 uptake rates are masking the lower rates of others. Therefore, except for the relatively undifferentiated embryonic stages in which distribution of activity was found to be equal, the O_2 uptake rates per cell must be viewed as average figures. Only as such do they have value when comparing the different developmental stages of embryogenesis.

The O_2 uptake studies and localization of enzyme activity support the idea that the tricarboxylic acid cycle is operating in all the stages of the embryo studied. Although no experimental evidence is available concerning the immediate stages following the first division of the zygote, aerobic respiration may be expected to occur here as well. This assumption is based upon the observation that the mitochondria have a normal appearance at all stages of embryogenesis and that the physical location of the developing embryo within the ovule is not one in which O_2 can be expected to be limiting.

Data from the inhibition studies lend further support to the idea that the tricarboxylic acid cycle is the only respiratory cycle operating in the stages studied.

Summary

Respiration rates as measured by oxygen uptake were determined for young stages of *Gossypium hirsutum* L. embryos, (globular through torpedo). Oxygen uptake increased as embryo size increased. However, when oxygen uptake was averaged on a per cell basis, the amounts decreased with development. Through the use of inhibition studies, characterization of the respiratory pathway was made. The tricarboxylic acid cycle appeared to be the only pathway involved in the oxidation of substrates to carbon dioxide. Cytochemical localization of succinic dehydrogenase activity revealed an association between enzyme activity and those parts of the embryo in which growth and differentiation were taking place. The shoot apex and other relatively quiescent portions of the embryo had characteristically low levels of associated enzyme activity.

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