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PHOTOCONTROL OF ALCOHOL, ALDEHYDE, AND ANTHOCYANIN PRODUCTION IN APPLE SKIN[1]

H. W. SIEGELMAN AND S. B. HENDRICKS

PIONEER RESEARCH LABORATORIES FOR PLANT PHYSIOLOGY AND MINERAL NUTRITION,
AGRICULTURAL RESEARCH SERVICE, U. S. DEPARTMENT OF AGRICULTURE,
BELTSVILLE, MARYLAND

While examining the influence of light on anthocyanin synthesis in pieces of apple skin floating on sucrose solutions, we noticed that light strongly inhibited the formation of the characteristic apple odor (10). In the present work the influence of visible radiation on aldehyde, alcohol, and anthocyanin formation was examined in detail. Radiation was found to suppress oxygen consumption markedly with accompanying sparing of sugar, suggestive of the Pasteur effect. One of the aromatic rings in the anthocyanin molecule is derived from acetate (1, 3, 4, 11), and anthocyanin synthesis in many plants requires radiation. The action spectra suggest that the photoreceptive pigment is the oxidized form of an acyl coenzyme A dehydrogenase (9, 10), in accord with the utilization of acetate. Further evidence that two photoreactions with the same photoreceptor are required for anthocyanin synthesis in apple skin is presented here.

MATERIALS AND METHODS

Early-harvested Jonathan and Arkansas apples were held at 0° C after being sealed in 1-bushel bags made of 0.38-mm polyethylene. Narrow strips of green areas of the apple skin were removed with a White Mountain apple peeler. The strips were cut into 7- × 7-mm pieces with razor blades mounted in a holder. The pieces, which had some cortical tissue attached, were washed twice with 0.3 M sucrose and then for one hour in running tap water. Fifty-one pieces (25 cm^2) of apple skin were placed cuticle side up in a 10-cm Petri dish containing 15 ml of 0.3 M sucrose. The Petri dishes were held in darkness or irradiated with a cool white fluorescent source of radiant energy, giving a maximum illumination of about 2000 foot-candles (ft-c).

All inhibitors and substrates used were dissolved in 0.3 M sucrose and the final solution was adjusted to pH 4. All temperatures reported were measured in the sucrose solution in which the pieces of apple skin floated.

In all experiments in which anthocyanin synthesis was examined, the Petri dishes containing the apple-skin pieces were held 24 hours in darkness after irradiation at a temperature three degrees lower than that during the irradiation period.

The anthocyanin content of the pieces of apple skin was determined on an extract made at 1° C in

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darkness from 25 pieces (12 cm^2) of apple skin in 10 ml of 1 % HCl in methanol. The absorbance measurements were converted to moles anthocyanin/cm^2 by the use of a molar-extinction coefficient (ϵ) of 3.43×10^4 at 530 mμ. The molar-extinction coefficient was determined on an authentic sample of cyanidin 3-galactoside (idaein).

The aldehyde and the alcohol production of the pieces of apple skin were determined enzymatically on a steam distillate of apple-skin pieces. All the pieces of apple skin and the sucrose solution from one Petri dish were quantitatively transferred to a 50-ml flask and steam-distilled until 25 ml of distillate was collected. The distillate was stored in sealed vials at 1° C until analyzed. The aldehyde content of the distillate was assayed enzymatically by a slight modification of the procedure of Racker (7). The following were placed in a 1-cm light-path pyrex-glass cuvette: 0.05 ml of 1 M potassium phosphate buffer, pH 7.2; 0.1 ml of 3×10^{-3} M DPNH (reduced diphosphopyridine nucleotide) in distilled water; and 2.75 ml of distillate containing aldehyde or distilled water. An initial optical-density reading was taken at 340 mμ (maximum for DPNH) and then 0.05 ml of a 1 % solution of crystalline yeast alcohol dehydrogenase (Worthington) in 0.1 % bovine serum albumin and 0.01 M potassium phosphate, pH 7.2, were added and the cuvette contents stirred. The reduction of the aldehyde was completed in about two minutes, as indicated by the constancy of optical-density readings. The final density reading was subtracted from the initial density reading and the aldehyde concentration was calculated by use of a conversion factor of 0.1 micromole aldehyde equals a density change of 0.21 at 340 mμ.

The alcohol content of the distillate was assayed by a modification of the enzymatic procedure of Bonnichsen and Theorell (2). Yeast alcohol dehydrogenase was found to be just as satisfactory as liver alcohol dehydrogenase for this assay at the levels of alcohol measured. The following were placed in a 1-cm light-path pyrex-glass cuvette: 0.1 ml of 1×10^{-2} M DPN (diphosphopyridine nucleotide) in distilled water; 1.0 ml or less of distillate containing alcohol; and a buffer-semicarbazide solution to make a total volume of 3.2 ml. The buffer-semicarbazide solution had the following composition: 3 vol. of 0.1 *N* NaOH + 7 vol. of 0.1 *N* glycine in 0.1 *N* NaCl + 1 vol. of 0.1 *N* semicarbazide hydrochloride in 0.1 *N* NaOH. An initial optical-density reading was taken at 340 mμ and then 0.05 ml of the yeast alcohol dehydrogenase enzyme was added and the cuvette contents stirred. The final optical-density reading was made after exactly 30 minutes at room temperature. Standard curves were determined for each series of analyses by use of the following amounts of alcohol: 0, 0.10, 0.20, 0.40, and 0.60 micromole. The initial optical-density reading was subtracted from the final value minus the blank, and the alcohol concentration was determined from the standard curve.

The alcohol dehydrogenase enzyme will react with several aldehydes, ethanol and higher aliphatic alcohols. Therefore, the values were expressed in this paper only as alcohol and aldehyde since the enzyme is not exclusively specific for acetaldehyde and ethyl alcohol. However, according to White (12), acetaldehyde accounts for 60 % of the carbonyl compounds and ethyl alcohol for 77 % of the alcohols in apple-juice volatiles.

Respiration measurements were made by standard Warburg manometric procedures with shaking at 80 cycles/minute. The 0.3 M sucrose in which the pieces of apple skin were placed was about 7 mm deep in the manometric vessel. During shaking in light some of the pieces had the cuticle side down or were obscured by others floating over them. Carbon dioxide assimilation was measured in Warburg vessels at 2 mm pCO_2 and a $C^{14}O_2$ activity of 30 μC/vessel.

Results

The time courses of alcohol and aldehyde formation in pieces of Arkansas apple skin in darkness or light were determined at several temperatures. Syntheses of these compounds were markedly influenced by both light and temperature (fig 1 a and 1 b).

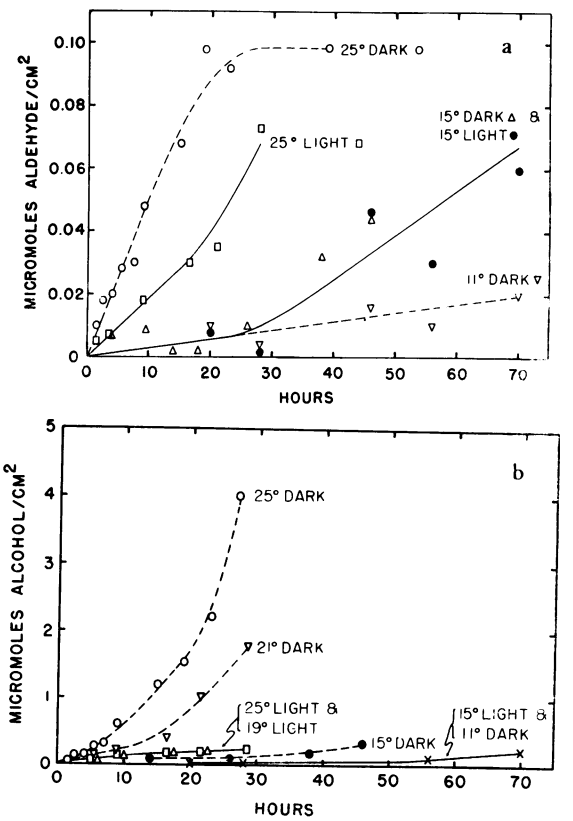

Fig. 1. Dependence of aldehyde and alcohol formation in pieces of skin of Arkansas apple floating on 0.3 M sucrose on the time of holding in darkness or light at the indicated temperatures: (a) aldehyde; (b) alcohol.

TABLE I

ANTHOCYANIN FORMATION IN PIECES OF ARKANSAS APPLE SKIN AFTER A 24-HOUR IRRADIATION PERIOD AT 21° C IN THE PRESENCE OF SEVERAL COMPOUNDS

COMPOUND	3×10^{-1}	MOLES ANTHOCYANIN $\times 10^{-8}$/CM^2 FORMED AT INDICATED CONCENTRATION				
		10^{-1} M	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M
Pyruvate	...	0.85 *	1.45	1.27	1.52	...
Shikimate	...	...	...	1.24	...	...
Acetate	...	...	...	0.99 *	1.20	1.52
Iodoacetate	...	...	...	0 *	...	...
Sucrose	1.24	...	...	...	...	...

* Injury evident.

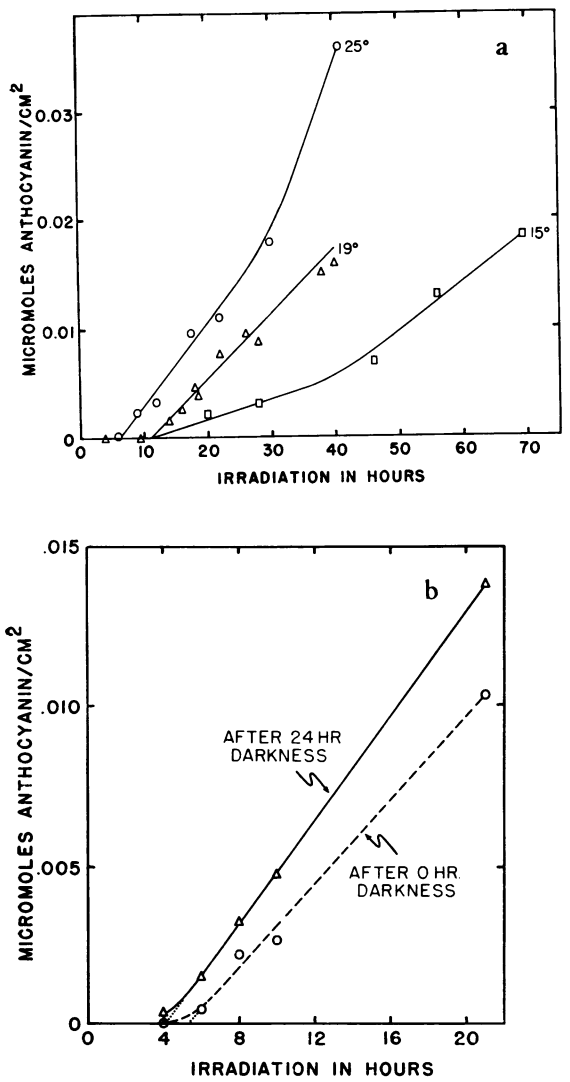

FIG. 2. Dependence of anthocyanin formation in pieces of apple skin floating on 0.3 M sucrose on the time of irradiation under a fluorescent light source: (a) pieces of Arkansas apple skin at the indicated temperatures; (b) pieces of Jonathan apple skin at 25° C with and without prior holding on the sucrose solution in darkness.

The time course of anthocyanin formation in pieces of Arkansas apple skin irradiated at several temperatures was examined. The rate of anthocyanin formation and the induction period before anthocyanin formation begins are both affected by temperature (fig 2 a). The time course of anthocyanin synthesis in pieces of skin of Jonathan apple held in the dark for 24 hours before irradiation was compared with that in pieces of apple skin irradiated immediately after preparation (fig 2 b). The temperature during this experiment was 25° C. There is considerable formation of apple volatile substances during the dark period. The standard errors for the anthocyanin contents after 21 hours of irradiation were 0.08×10^{-8} and 0.07×10^{-8} moles anthocyanin/cm^2 for samples held in darkness and not held there, respectively. The corresponding standard errors after 8 hours of irradiation were 0.012 and 0.004×10^{-8} moles anthocyanin/cm^2.

Possibly stimulatory or inhibitory effects of several substances on anthocyanin synthesis were examined. Jonathan apple skin pieces formed 0.99, 0.88, 0.81, and 0.88×10^{-8} moles anthocyanin/cm^2, respectively, in 0, 0.014, 0.028, and 0.056 M ethanol after a 24-hour irradiation period at 25° C, with a standard error of 0.12×10^{-8} moles anthocyanin/cm^2. Anthocyanin production in pieces of Arkansas apple skin after a 24-hour irradiation period at 21° C in the presence of various compounds is shown in table I. Injury, seen as discoloration of the skin pieces, was evident in the 10^{-1} M pyruvate and 10^{-3} M acetate solution. Phosphate at 10^{-3} M did not influence aldehyde or alcohol production.

The fresh weight of the Arkansas apple skin tissue was 0.084 g/cm^2. This weight includes a considerable amount of attached cortex tissue. The cortical tissue held at 21° C in darkness and at 25° C in light did not produce significant amounts of aldehyde in 24 hours. The alcohol production of the cortical tissue is about 10 % that of the skin tissue.

Anthocyanin formation is first evident at the edges of the pieces. This is obviously a wound reaction not eliminated by washing.

The respiration, measured as O_2 uptake and CO_2 production, of pieces of Arkansas apple skin held on 0.3 M sucrose for 24 hours at pH 4 was as follows:

	O_2 Uptake moles $\times 10^{-8}/cm^2/hr$	CO_2 Production moles $\times 10^{-8}/cm^2/hr$
Light	16.0	19.0
Dark	26.5	32.3

The ratios of CO_2 production to O_2 uptake (R.Q.) were 1.19 and 1.22 in light and dark, respectively. Apparent respiration of freshly prepared pieces of green Arkansas apple skin floating on 0.3 M sucrose was reduced to zero by 1000 ft-c illumination. The extent to which this apparent suppression of respiration was a result of photosynthesis was not determined. The photosynthetic rate of the irradiated pieces was about one-tenth that of a young soybean leaf as measured by $C^{14}O_2$ uptake. An apparent zero respiration on irradiation would imply a very effective recapture of respiratory CO_2 by photosynthesis despite the presence of 20 % KOH in the center well of the Warburg vessel and low illumination of some pieces.

Discussion

Radiation is strikingly effective in controlling metabolism in the apple skin. It is necessary for anthocyanin synthesis and strongly inhibits alcohol formation. Oxygen consumption is also possibly inhibited in light and sugar is spared. The effects are clean-cut for alcohol and anthocyanin syntheses.

Inhibition of alcohol formation by radiation is most evident at 25° C but is still seen at 21° and 15°. Alcohol, anthocyanin, and aldehyde production, O_2 uptake, and CO_2 production per hour during the first 20 hours at 25° C in 1 cm^2 of Arkansas apple skin were as follows:

	Dark moles $\times 10^{-8}/cm^2/hr$	Light moles $\times 10^{-8}/cm^2/hr$
Alcohol	8.5	1.0
Aldehyde	0.5	0.2
Anthocyanin	0.0	0.8
O_2 (uptake)	26.5	16.0 (apparent)
CO_2 (production)	32.3	19.0 (apparent)

The alcohol production, calculated from the difference between O_2 uptake and CO_2 production in the dark, would be 5.8 moles $\times 10^{-8}/cm^2/hr$ in comparison with the observed value of 8.5 moles $\times 10^{-8}/cm^2/hr$. The discrepancy is very likely due to the markedly different conditions for measurement of the two values. Either value, though, indicates that approximately equal quantities of sugar are fermented and oxidized in darkness.

The alcohol production of the pieces of apple skin in light is reduced to 10 % of the value in darkness or to 15 % if aldehyde is included. The apparent oxygen uptake is reduced about one-half, but it is probably confounded with evolution of oxygen in photosynthesis. A similar suppression of apparent respiration was noted by Siegelman et al (8) for immature rose petals, but possible confounding with photosynthesis would have to be carefully considered.

Aldehyde is an intermediate in alcohol formation. In darkness it presumably would be produced at a steady-state rate depending upon its rate of formation from pyruvate and its reduction to alcohol. The fact that aldehyde does not markedly accumulate in light indicates that the suppression of alcohol formation is controlled at or prior to pyruvate utilization.

The formation of anthocyanin, $C_{15}H_{11}O_6$ for cyanidin, from sugar would not require CO_2 evolution or oxygen consumption. The synthesis of 0.8 moles $\times 10^{-8}$ anthocyanin/cm^2/hr in apple skin would require as much sugar as 4 moles $\times 10^{-8}/cm^2/hr$ of alcohol formation in darkness. The pieces of apple skin actually produce 8.5 moles $\times 10^{-8}/cm^2/hr$ of alcohol in darkness. A material balance for anthocyanin and alcohol indicates a sparing of sugar in light, similar to the effect of oxygen in the Pasteur effect. The way by which sugar is spared in the Pasteur effect is unknown. The proposed explanations are discussed by Laties (6) and the difficulties with their acceptance are given by Krebs (5).

The tentative conclusions which can be drawn from the time courses of the appearances of the several products are now considered. Time is required for each product to reach a steady-state rate of formation after the apple skin pieces are placed in a sucrose solution. This is less than 0.5 hour at 25° C for aldehyde (fig 1 a) in harmony with a probable high turnover rate for pyruvate. The rate of alcohol accumulation (fig 1 b), on the other hand, increases steadily as would be expected for an end-product dependent on prior aldehyde production. Anthocyanin formation has an induction period with a lag of at least 6 hours at 25° C. About one-fourth (1.5 hours) of this induction period is eliminated by allowing the apple skin pieces to float on a sucrose solution in darkness prior to irradiation (fig 2 b). The 1.5-hour reduction in the lag period is the time required for the formation of the 1st substrate, which requires light for conversion to a 1st light product of anthocyanin formation. An additional 4.5 hours is required before radiation induces anthocyanin formation. This period is the time required for the 1st product of light action to start forming the substrate for a 2nd light action. A steady state of anthocyanin formation is then attained in about one additional hour. The schematic course of anthocyanin formation can now be conceived as follows, with the approximate time required at 25° C for the reaction shown above the arrow to approach steady-state conditions or to reach essential completion:

Sucrose $\xrightarrow{\text{30 minutes}}$ pyruvate $\xrightarrow{\text{1 hour}}$ active acetate

$\xrightarrow[\text{radiation-limited step number 1}]{}$ first product of radiation

$\xrightarrow{\text{4 hours}}$ intermediate reactions including "head to

tail" active acetate condensation product of radiation $\xrightarrow{\text{radiation-limited step number 2}}$ second product of radiation $\xrightarrow{8 \text{ hours}}$ dark reactions leading to eventual cyclization of a polyacetate compound into the phloroglucinol ring of anthocyanin. The active acetate compound involved in the 1st photoreaction reaches a steady-state condition limited by the utilization of active acetate in respiration through the tricarboxylic acid cycle. That the same photoacceptor is involved in both photoreactions was indicated in the previous work on the action spectrum (10). The time courses of anthocyanin synthesis in seedlings of turnip and red cabbage (9) support these deductions. Turnip seedlings grown in the presence of chloramphenicol have already reached the steady-state formation of the product of the 3rd reaction in darkness. The turnip seedlings show no evidence of the 1st two reactions of the scheme. Red cabbage seedlings, after the photomorphogenic condition is established, have an immediately linear time course of anthocyanin synthesis. The 1st five steps are already at steady state in red cabbage seedlings and only the 2nd photoreaction appears. The red cabbage seedlings can thermally accomplish the 1st photoreaction and also the 2nd, as shown by the appreciable synthesis of anthocyanin in darkness.

Summary

The time courses of aldehyde, alcohol, and anthocyanin syntheses in light and darkness were measured in apple skins floating on sucrose solutions. Radiation suppresses aldehyde and alcohol syntheses with a sparing of sugar in a manner equivalent to the suppression of fermentation by oxygen and sugar use in fermentative systems. The time course of anthocyanin synthesis indicates that two radiation-limited steps are involved in anthocyanin synthesis in apple tissue.

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LIPID SOLUBLE PIGMENTS OF WHEAT PLANTS AS RELATED TO HESSIAN FLY INFESTATION[1,2]

BRYON S. MILLER, HOWARD L. MITCHELL, JOHN A. JOHNSON AND ELMER T. JONES

Crops Research Division, A.R.S., U.S.D.A.; Department of Chemistry, Kansas Agricuctural Experiment Station; Department of Flour and Milling Industries, Kansas Agricultural Experiment Station and Entomology Research Division, A.R.S., U.S.D.A.

The dark blue-green color of the center leaves of certain wheat plants is the visual means whereby experienced entomologists select hessian fly infested wheat or barley plants. This color is abnormal because center leaves are younger than outer ones and when uninfested are light green in color. In normal plants, the older, outer leaves are darker green than the younger center leaves. Differences in type or concentration of plant pigments may explain these differences.

The objective of this study was to investigate the possible relationship of pigmentation in hessian fly

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