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STUDIES ON THE ETHYLENE PRODUCTION OF APPLE TISSUE[1, 2, 3]

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Ethylene has been identified as a normal metabolic product of a wide variety of fruits (1, 15, 24), many flowers (6, 10, 29), certain fungi (7, 26, 40) and some leaves (6, 14, 31). Although studies of the biological effects of ethylene have revealed that in trace amounts ethylene is able to modify a number of processes of growth and development, the gas is normally produced in such minute quantities that physiologically active concentrations seldom accumulate. Flowers, for instance, respond to their endogenously produced ethylene only when they are confined without ventilation (10). Ripe fruits, however, are an exception in that their tissues may contain large internal concentrations of ethylene which are sufficient to accelerate the ripening of immature fruits. It is uncertain whether the gas normally initiates ripening, i.e. acts as a ripening hormone, or whether it is simply a by-product of the process. There is general agreement, however, that ethylene production is restricted to that stage in the life of the fruit during which ripening occurs. In fact, the time at which this substance begins to be produced is very near to the time of onset of fruit maturation. A knowledge of the chemistry of ethylene production therefore, should help to elucidate the metabolic changes which take place just prior to ripening. Moreover, should ethylene truly initiate natural ripening, it would be all the more important to know how the gas is synthesized and under what conditions the synthesis is initiated.

Although there are a few reports describing the preparation of enzyme extracts which are capable of producing ethylene (7, 12, 13), attempts to duplicate these results by the original methods and by other procedures have so far been unsuccessful. For this reason studies were undertaken with tissue sections. The apple was chosen as an experimental material because of its relatively high rate of ethylene production. In the past, research on the biosynthesis of ethylene has been hampered by lack of a sufficiently sensitive and specific quantitative assay, but the recent development of gas chromatography has circumvented this problem and made possible a new approach. This paper reports some of the results which have been obtained using gas chromatography to investigate the ethylene production of apple tissue sections.

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Materials and Methods

McIntosh apples were purchased at the local market and stored at 4° C. The apples were found to attain their maximum rate of ethylene production about 10 hours after they were transferred from cold storage to room temperature (4). The apples were, therefore, used for experiments at that time. A few experiments were carried out with Baldwin and Red Delicious apples, and with Bosc pears, all of which were treated in about the same way.

The construction and operation of the katharometer and gas chromatography system have been described in detail elsewhere (2, 3). The sample of gas was injected into a flowing stream of helium which passed through an 18 in. x 1/4 in. tube containing aluminum oxide. The gases, emerging from this chromatography column as separate bands, were quantitatively assayed by means of a katharometer of special design. Apple emanations were characteristically fractionated into two separate bands on the aluminum oxide; the first contained oxygen, nitrogen, carbon dioxide and perhaps trace amounts of apple volatiles; the second contained only ethylene. Less than 10^{-2} μl of ethylene could be accurately measured by this procedure.

Several independent means were used to establish that all of the material which was measured on the chromatograms was ethylene. Nitrogen, oxygen, methane, ethane, carbon dioxide, ethylene, propylene, *n*-butane, isobutane, ammonia, nitrous oxide, acetylene, hexane, carbon monoxide, water vapour, ethyl alcohol vapour and acetaldehyde were tested for chromatographic behavior on the aluminum oxide, and only synthetic ethylene was found to have the same retention time as the substance contained in apple emanations. Nitrous oxide chromatographed only slightly ahead of ethylene and mixed chromatograms of the two gases showed a single peak. The two gases were separated, however, on a long silica gel column and apple volatiles showed only a single peak corresponding to ethylene when this adsorbent was used. The material was completely absorbed in bromine water and on mercuric perchlorate reagent (41); it could be recovered quantitatively from the latter reagent by addition of HCl or lithium chloride (see 2 for exact procedure). Since the mercuric perchlorate reagent is specific for olefines, and other olefines, if present, would have given clearly separate bands, the identification of ethylene is definite.

Although only a few ml of gas could be passed through the chromatography column in a single analysis, it was found that the volume of air in contact with a few grams of tissue could readily be kept to this minimum. This allowed direct measurement of the ethylene. About 12 plugs, 4x1 cm in size, were cut from a single apple with a cork borer. The plugs were rinsed in tap water, dried, weighed (ca 2.7 g each), and inserted in 5 ml hypodermic syringes into which they fitted very closely. Silicone lubricant, in which ethylene is not soluble, was used to grease the syringe plungers, which were then set at the 4.2 ml mark. Since previous experiments had shown that evacuation does not affect the subsequent rate of ethylene production (4), all syringes were placed in a desiccator and evacuated to remove the ethylene present in the intercellular spaces. Immediately after air was readmitted needles were added and sealed by insertion into rubber stoppers. The syringes were then incubated for 60 to 90 minutes at 27° C. This temperature was found to be close to the optimum for ethylene production in McIntosh apples (4). At the end of the collection period the stoppers were removed, the contents of the syringes were pressed out and the gases were collected over water. Since apple tissue is soft enough to be pressed out of a syringe, and ethylene has only a very slight solubility in water, almost the entire gaseous contents (ca 1.8 ml) as well as the contained ethylene (ca 0.2 μl) were recovered. The entire sample was analyzed by gas chromatography, which yielded a value for the concentration of ethylene in the atmosphere surrounding the tissue. The total volume of air initially present in the syringe was calculated from the known volume of the syringe (4.2 ml) and the weight of the contained tissue, using the facts that apple tissue has a density of 0.8 (24) and a free air space of 33 % (25). When this procedure was employed, it was found that determinations of the rate of ethylene production of apple sections extracted from various parts of a single fruit agreed within ± 5 %.

The oxygen consumption and carbon dioxide production of tissue sections (ca 1 g weight) were determined in constant volume respirometers. A differential system with and without alkali was used for carbon dioxide measurements (39).

Results

Area of Cut Surface: Plugs cut from various parts of an apple showed nearly identical rates of ethylene production, and they maintained these rates with very little decline for at least 7 hours after the tissue had been removed from the fruit. Changes in the cut surface area, however, markedly affected respiration and ethylene production in apple tissue. Table I illustrates the progressive rise in respiration and decline in ethylene production which occurred

Table I.

Effect of Cutting on Respiration and Ethylene Production of McIntosh Apples at 23° C

	Whole* apple	4 cm plugs**	1 mm slices**
Ethylene (μl/Kg/hr)	97.5	95.2	41.0
Carbon dioxide (μl/g/hr)	17.1	33.5	48.5
Oxygen (μl/g/hr)	15.2	21.7	28.8
Area of cut surface (cm²/g)	0	5.8	33.3

* The ethylene production and respiration rate of the whole fruit were determined by methods somewhat different from those used for the tissue sections. Details are given elsewhere (2, 11).

** Measurements over a one hour period after cutting.

as the cut surface area was increased. There is evidence that the increased evolution of carbon dioxide is the result of the decarboxylation of malate (20, 22), but the changed oxygen consumption and ethylene production cannot be immediately related to malic acid metabolism. The ethylene output of 4 cm plugs was not significantly different from that of the intact fruit, but that of the 1 mm tissue slices, in which the cut surface area was five to six times greater, was reduced by 58 %. As the slices averaged 10 to 12 cells in thickness the effect cannot be accounted for solely by physical damage at the cut surface.

In searching for a convenient compromise between the intact plugs (into which solutions could penetrate only with difficulty) and the tissue slices with their diminished rate of ethylene production, it was found that the ethylene production was reduced less than 10 % when a 1 mm hole was bored longitudinally through the center of an apple plug and the 1 mm center cylinder replaced in it. The permeability of plugs prepared in this manner was compared to that of tissue slices by soaking both plugs and slices in various concentrations of iodoacetamide and determining the oxygen consumption. The penetration of the inhibitor, as judged by the decline in respiration at the various applied concentrations, was found to be identical in the two cases.

WATER SOAKING: When plugs were prepared as above by boring holes through their centers, and both the resulting pieces were soaked in water for 60 minutes at room temperature before they were dried and reassembled, the tissue consistently showed a 50 to 75 % decrease in ethylene production as compared to unsoaked plugs prepared in a similar manner. A comparable decrease was found when 1 mm thick slices were soaked in water. This effect was not due to a water soluble inhibitor, since water in which tissue had been macerated was no more inhibitory than distilled water or continuously changed tap water. The respiration rate of both slices and plugs was unaffected when the tissue had been soaked in water for 60 minutes.

One possible explanation for the decrease of ethylene production caused by soaking in water is that essential materials escape from the sections. An experiment was designed to measure the actual loss of two classes of compounds, namely reducing sugars and acids. Six plugs were cut from an apple. Holes were bored through their centers and the plugs were rinsed, dried and weighed. Three plugs were at once individually extracted by grinding in a mortar with 50 % ethanol. The solutions were filtered, the alcohol removed under reduced pressure and the volumes adjusted to 50 cc. The remaining three plugs were placed in individual Petri dishes, each containing 50 cc of distilled water. After one hour the plugs were removed and extracted with ethanol as before. A 10 cc aliquot of each sample was purified by warming with a few drops of lead acetate, filtering, adding a crystal of dibasic phosphate and filtering again. The reducing sugar content of this final solution was determined by Nelson's arsenomolybdate method (24). A 35 cc aliquot from the original solution was used for the determination of the total acid content. This aliquot was titrated to pH 9.0 with carbon dioxide free base, and then back-titrated to pH 3.0 with standard 0.1 *N* HCl; distilled water was titrated to similar values as a control. The results (table II) show that apple tissue is remarkably permeable. It loses about 17 % of its reducing sugar and 25 % of its total acidity during one hour's soaking in water.

TABLE II.

CHANGES IN REDUCING SUGAR AND TITRATABLE ACID CONTENT OF APPLE PLUGS*

	REDUCING SUGAR (G/KG FW OF TISSUE)		
SAMPLE	UNTREATED TISSUE**	TISSUE AFTER SOAKING	WATER
1	53.7	44.8	9.6
2	49.9	47.8	9.8
3	55.7	46.3	9.1
Av.	53.1	46.3	9.5
	Acidity (meq/Kg FW of tissue)		
Sample	*Untreated tissue***	*Tissue after soaking*	*Water****
1	88.2	59.2	22.3
2	87.0	58.6	19.8
3	87.6	63.8	16.3
Av.	87.6	60.5	19.5

* Soaked 60 minutes in distilled water.

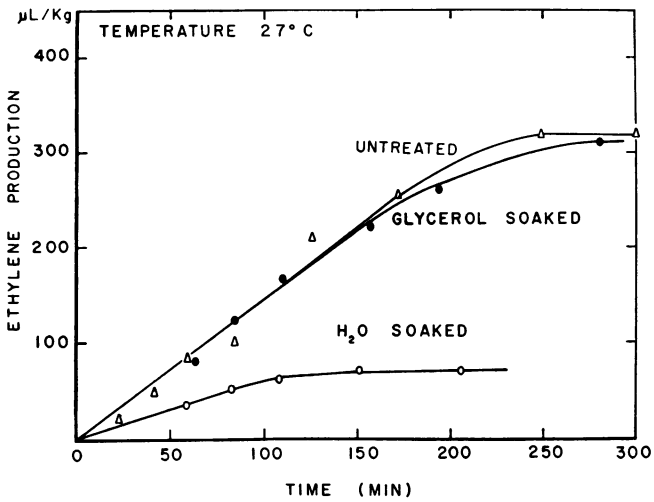
** Untreated tissue samples are arranged in an arbitrary order.

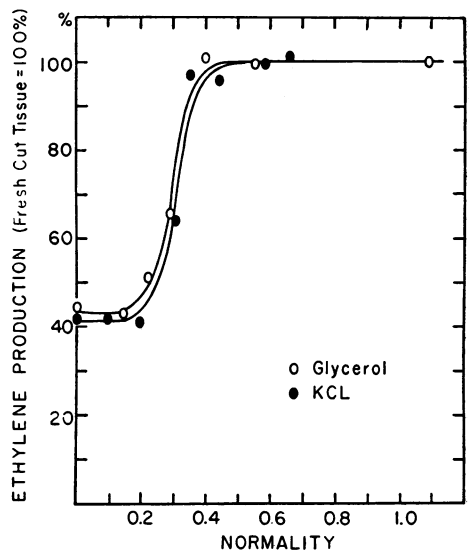
*** The actual amount of acid which escaped from the tissue must be greater than that recovered in the water since applied malate is rapidly decarboxylated (22).

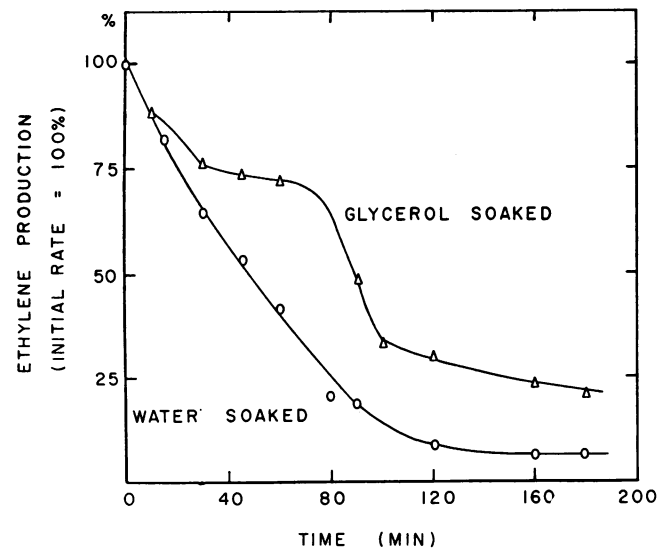
If the decline in the ethylene production of tissue soaked in water is due to the escape of solutes from the cells, it might be expected that this effect could be reversed, or the ethylene production accelerated, by adding compounds which could replace the lost material. Accordingly, various compounds were soaked into apple plugs prepared as above. A number of these were found to be very effective but, as will be seen, the basis of their activity is entirely different from that which might be expected.

EFFECT OF VARIOUS SOLUTES IN MAINTAINING ETHYLENE PRODUCTION: Unless otherwise stated the soaking in water or test solution was of one hour's duration in all cases, and the two pieces were then reassembled and their rate of ethylene production determined. One of the first compounds to be tested was glycerol. Its effect will be discussed in detail since it was used as a standard.

Figure 1 presents a typical experiment. After the tissue was soaked in water for 60 minutes its rate of ethylene production was greatly decreased. The low rate continued more or less unchanged for about 100 minutes and then fell almost to zero. If instead, the tissue were initially soaked in 0.55 M glycerol solu-







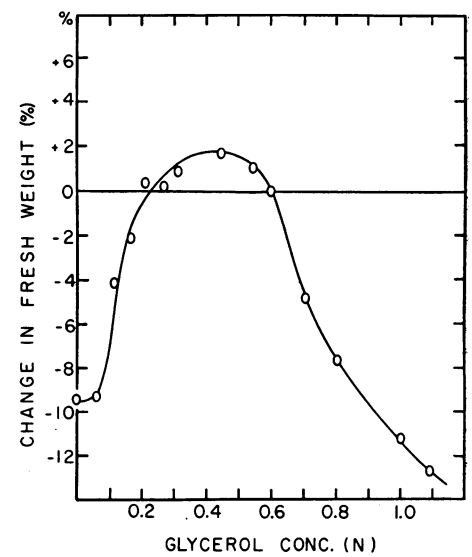


FIG. 1 (*upper left*). Effect of pretreatment on the subsequent rate of ethylene production. Lower curve: tissue soaked in water for 60 minutes. Center curve: tissue pretreated with 0.55 M glycerol for 60 minutes. Upper curve: untreated controls. Each curve the mean of 6 to 10 experiments.

FIG. 2 (*upper right*). Relative effectiveness of various concentrations of glycerol and potassium chloride in maintaining the ethylene production of apple tissue sections. The solutions were soaked into the tissue for 60 minutes and the ethylene production was determined during the next 90 minutes. The KCl curve is corrected for association which occurs at the higher normalities.

FIG. 3 (*lower left*). Time-course curves showing the ability of glycerol to reverse the effect of water soaking. In both curves the time corresponds to the total duration of the initial water soak. At the times indicated two apple plugs were removed from water. Lower curve: one sample was tested for ethylene production during the next 1 hour interval. Upper curve: the second sample was soaked in 1 M glycerol solution for 30 minutes before its rate of ethylene production was determined.

FIG. 4 (*lower right*). Changes in the fresh weight of apple cylinders after 60 minutes treatment with various concentrations of glycerol.

tion, its rate of production of ethylene remained close to that of fresh untreated controls, and furthermore, the production continued linearly for the same length of time as in controls. The data presented here were derived from samples which were all placed in syringes at the beginning of the experiment. Both the glycerol treated and the untreated tissues ceased to make ethylene after 250 minutes of confinement, but calculations based on the respiration rate and the free air space of the syringes show that this is exactly the time at which the oxygen content should have dropped to between 1 and 3 %. Ethylene production is strongly inhibited by these low oxygen partial pressures. The process, in fact, ceases when the conditions become completely anaerobic (4, 15). It seems in general that glycerol produces an effect of very long duration; as nearly as can be determined, glycerol maintains the tissue in very nearly its normal metabolic condition.

The ethylene production rates in a series of experiments at different concentrations of glycerol are plotted in figure 2. In each case the steady rate after 60 minutes soaking is expressed as percentage of that of the fresh control tissue in the same experiment. It is evident that at concentrations above 0.4 M, glycerol completely prevents the decline in ethylene production and maintains the rate at essentially that of fresh cut tissue. This was true even at above 1 M, when the apple plugs were completely flaccid. In no instance, however, was the rate increased above that of the fresh tissue. The respiration rate of the glycerol treated tissue remained close to that of the controls in all cases.

The effect on ethylene production and respiration exerted by a variety of other organic substances was investigated in order to determine whether a substrate specificity was involved. Glycerol was assigned the value of 100 % activity. In each experiment rates were determined for tissue treated with 5 % (0.55 M) glycerol, water and a 5 % solution of the test material (adjusted to pH 5.0). Many compounds were found to prevent to varying extents the decline in ethylene production induced by water soaking. In addition to glycerol, hydracrylic amide proved to be 100 % effective; Sodium pyruvate, potassium malate, acetamide, malonamide, glycolamide, ethanolamine-hydrochloride, lactamide, glycine, alanine and sodium 2-phosphoglycerol were between 50 and 90 % effective. Several sugars and sugar phosphates (sodium salts), sodium citrate, choline hydrochloride, ethylene glycol and 1,2-propanediol produced smaller though definite effects. Most of the other substances tested, including a large array of organic acid salts, alcohols and nitriles, were equally inhibitory to both ethylene production and respiration even at relatively low concentrations. In fact, with minor exceptions, all compounds which when present at high concentrations did not disrupt the tissue respiration were at least partially able to reinstate ethylene production. In no case was a compound found to show any activity at a concentration below 0.2 M. This fact along with the obvious lack of metabolic relationship between many of the substances, suggests that the various materials are not serving as substrates for ethylene synthesis.

If a substance necessary for ethylene production leaks out of apple tissue when it is placed in water, then glycerol and the many other active organic compounds might in some way alter the permeability of the tissue and prevent this material from escaping. If this were true, then glycerol applied after tissue had been water soaked, should not be able to reinstate the ethylene production. For 100 minutes (fig 3) a nearly linear relationship exists between the duration of the exposure to water and the extent to which the ethylene production falls off. If at any time during the first 80 minutes the tissue was removed from water and soaked for 30 minutes in 1 M glycerol, the ethylene production was almost completely reinstated. Even after 80 minutes, glycerol always produced approximately the same percentage acceleration but the tissue had gradually lost much of its ability to produce ethylene. Similar results were obtained with hydracrylamide. Since both of these compounds increased the ethylene production *after* water soaking had lowered it, it follows that the compounds could not have acted by preventing a substance from leaving the tissue. On the contrary, they must either themselves penetrate into the tissue or else cause an osmotic withdrawal of water. Moreover, the damage induced by water soaking eventually became irreversible. This strongly suggests that an enzyme system is being affected by the treatment.

Nature of Change Caused by Soaking: As mentioned above, the fact that glycerol and other solutes are only effective in high concentrations suggests that the action may depend upon regulation of the cell's water content or solute concentration. This supposition was verified by the effectiveness of certain inorganic compounds. Two salts, $CaCl_2$ and KCl, have been tested; 3 % (0.37 M) $CaCl_2$ was about 50 % as effective as glycerol in maintaining the ethylene production, whereas 3 % (0.4 M) KCl was just as active as the best glycerol concentrations. In figure 2 the relative activities of various normalities of glycerol and potassium chloride are compared. Equivalent normalities of both substances are equally effective. This indicates that the two compounds are almost certainly acting by the same mechanism, and the mode of action must therefore be osmotic.

In storage tissue such as the apple, the changes in water content can be studied by carefully drying and weighing tissue sections before and after treatment with inert solutes. The effects of externally applied glycerol solutions and water on the water content of apple plugs were therefore studied in this way. The main object was to determine whether it is the water content or the solute content of the cells which is critical for ethylene production. When apple plugs were soaked in water, it was found that the osmotic response of the tissue was atypical. Water soaking (table III) did not bring the tissue to full turgor, except perhaps for the first few minutes; there was, instead, a steady loss in fresh weight for

TABLE III.

CHANGES IN WATER CONTENT OF APPLE TISSUE SOAKED IN WATER AS A FUNCTION OF DURATION OF TREATMENT

DURATION OF TREATMENT (MIN)	% CHANGE IN TISSUE FRESH WEIGHT
2	0.0
5	+2.6
9	−1.3
10	−1.6
15	−5.7
30	−7.1
45	−8.4
60	−9.7
90	−10.4

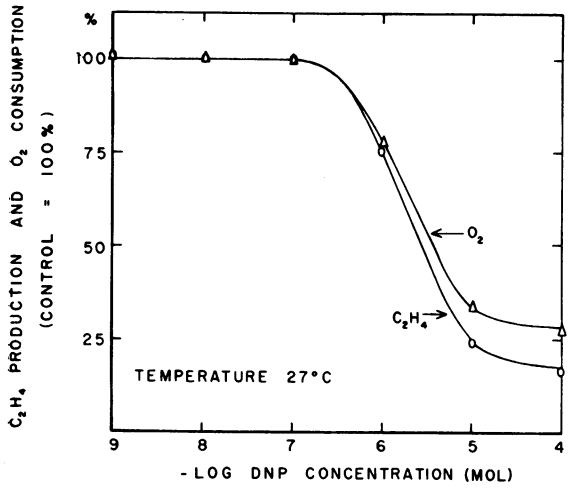
at least 90 minutes. After 1 hour the total loss in fresh weight was approximately 10 %. By this time the tissue had lost much of its turgor. The data of table II however, showed that in 60 minutes the leakage of solute from the tissue amounted to only about 1.8 % of the initial fresh weight. The conclusion is inescapable that the cells actually lose water when they are surrounded by water! From the figures in the table, it can be estimated that the solute decreased by about 17 %, whereas subtracting the weight of solute lost from the decline in tissue fresh weight yields a value for the loss in tissue water of 7.9 %. The solute concentration of the cells must therefore diminish by about 10 % in spite of the water loss. A measurement of the tissue's suction pressure, using mannitol as a plasmolytic agent, showed that it was nearly 0.65 M. Presumably the osmotic concentration is slightly higher than this figure, so that the net result of soaking apple tissue in water must be to diminish the solute concentration by at least 0.07 M.

Figure 4 presents the changes in weight which occurred when apple plugs were soaked in glycerol solutions of various concentrations. In the range 0.23 to 0.6 M glycerol, the fresh weight of the tissue, instead of declining, actually increased slightly. This result cannot be accounted for solely in terms of the weight of glycerol that entered into the cells. Even in complete equilibrium with 0.5 M glycerol, the fresh weight of apple tissue would increase by less than 4 %, whereas in water the observed loss was very nearly 10 %. In part, this peculiar response may be explained in terms of known physical laws. The amount of glycerol that penetrated into the tissue should be roughly proportional to the concentration of the applied glycerol solution. At low molarities the solute leakage was probably more rapid than the glycerol entry, causing the turgor pressure to decline and water to be forced out by the cell walls. At progressively higher glycerol concentrations an increased amount of glycerol permeated the cells; a point was reached at which the amount of glycerol which had entered exactly balanced the amount of solute which had escaped, so that the tissue reached optimum turgor. At still higher concentrations enough glycerol may have entered to increase the weight of the tissue slightly. Finally, at concentrations above about 0.6 M, plasmolysis would be expected to occur and, indeed, the tissue was found to be flaccid.

McIntosh apples are not unique in the behavior described above. In preliminary experiments, Red Delicious apples have been found to behave very similarly; Baldwin apples and Bosc pears also showed a sharp optimum in their fresh weight at a particular applied concentration, although they did not change in weight when soaked in water alone. One aspect of Bosc pear behavior suggests that the interpretation offered above cannot be the entire explanation. The osmotic pressure of the pear tissue was considerably higher than that of apples; plasmolysis did not occur until concentrations of sucrose higher than 0.95 M were applied. Pear sections reached their highest fresh weight when they were soaked in 0.6 M sucrose; the actual increase in weight amounted to more than 8 %, which is clearly too high to be accounted for by the weight of the sucrose which might have entered. Apparently, therefore, some water entry must also be involved.

Comparing the data shown in figures 2 and 4 provides convincing evidence that the ethylene-producing system is sensitive to the solute concentration, but not to the water content of the cell. All glycerol and KCl concentrations above 0.4 *N* (fig 2) are 100 % effective in preventing or reversing the decline in ethylene production caused by water soaking. A 0.4 *N* glycerol solution, however, left the tissue fully turgid. Indeed, it counteracted the loss that would have occurred in water, whereas a 1.09 *N* concentration produced an even greater water loss than did water alone. It follows that ethylene production and the water content of the cell cannot be directly related. If, on the other hand, the final tonicity of the cell sap is proportional to the concentration of the applied solution, then there could be a definite correlation between solute concentration and ethylene production. Thus, when the tissue was soaked in water, both the cellular solute content and the rate of ethylene production were decreased. When the tissue was soaked in a strong solution of glycerol or KCl, both the solute concentration and the ethylene production were maintained. The latter was at almost the same rate as that of untreated tissue. If this is the maximum rate of ethylene production of which the tissue is capable, then it is not surprising that above some critical level (0.4 *N*), increasing the concentration of glycerol or of KCl should produce no further increase in the ethylene production.

The ethylene production of other tissues behaved similarly to that of the McIntosh apples. Both Baldwin and Red Delicious apple sections showed very low rates of ethylene production when they were soaked in water, whereas the rates were maintained at a high level when the soaking was carried out in 0.55 M glycerol. After one hour's soaking in water, cylinders cut from Bosc pears consistently showed no detectable ethylene production at all. The production could be maintained if the tissue was soaked in sucrose solu-


C_2H_4 PRODUCTION AND O_2 CONSUMPTION (CONTROL = 100 %)
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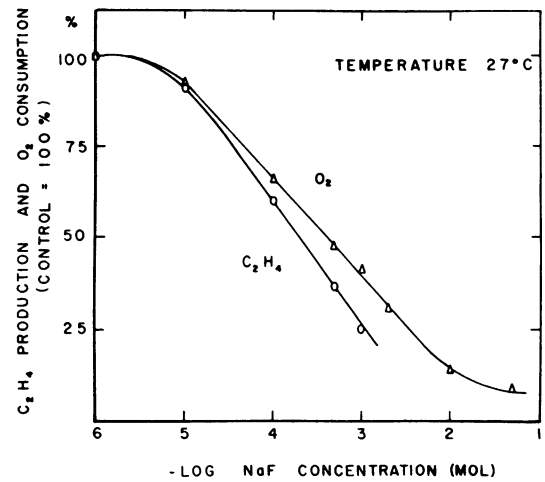
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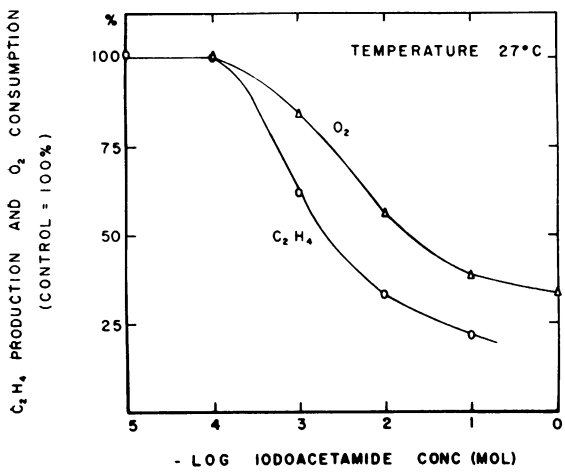
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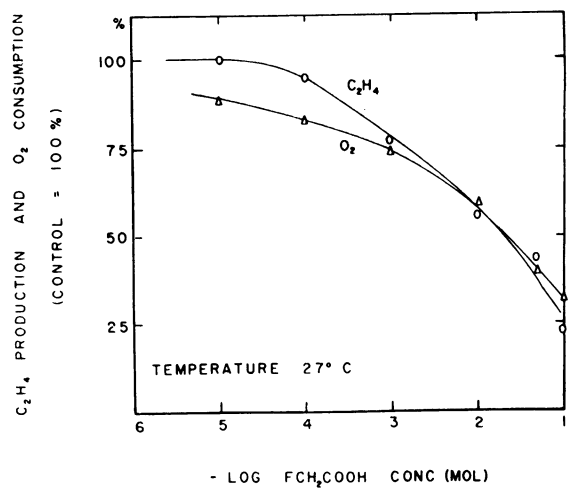
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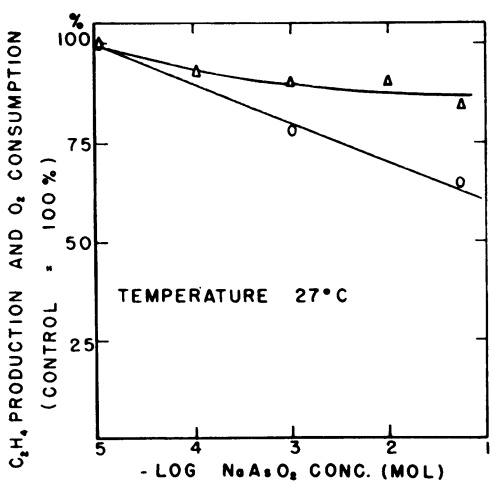
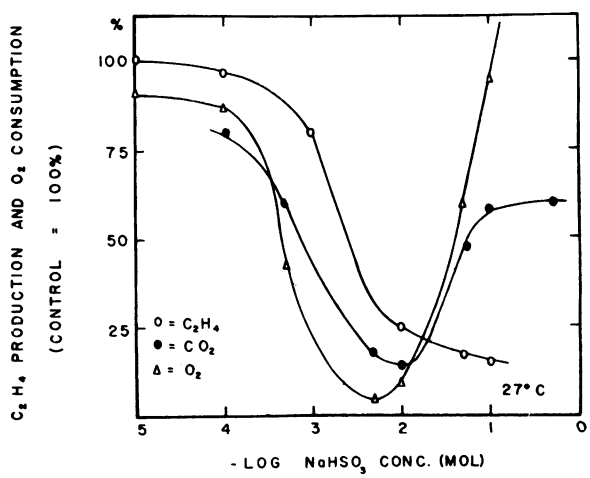
- LOG FCH_2COOH CONC (MOL)


C_2H_4 PRODUCTION AND O_2 CONSUMPTION (CONTROL = 100 %)
%
100
75
50
25
TEMPERATURE 27°C
5 4 3 2 1
- LOG $NaAsO_2$ CONC. (MOL)


C_2H_4 PRODUCTION AND O_2 CONSUMPTION (CONTROL = 100%)
%
100
75
50
25
o = C_2H_4
● = CO_2
Δ = O_2
27°C
5 4 3 2 1 0
- LOG $NaHSO_3$ CONC. (MOL)

tion; a rate equal to that of the freshly cut control was reached at about 0.9 M sucrose and above. Only at concentrations above 1.4 M was a definite decline noted.

INHIBITORS: Nine different inhibitors were investigated, using a range of concentrations soaked into McIntosh apple tissue plugs. It should be noted that in such a procedure depressing the rate of ethylene production by water soaking is unavoidable, and the effect of the inhibitor always is superimposed. This is not a severe limitation because water-soaked tissue from a single apple shows reproducible rates of ethylene production. These rates, therefore, provide acceptable control values. Figures 5 through 10 illustrate the changes in respiration and ethylene production induced by various concentrations of 2,4-dinitrophenol (DNP), fluoride, iodoacetamide, arsenite, fluoroacetate and sodium bisulfite. Thiourea and cyanide were non-inhibitory while not only was malonamide non-inhibitory, but also it was actually metabolized (decarboxylated) by the tissue.

Although the role of phosphorylation in mature fruits and in the ripening process has been the subject of dispute (21, 28, 34), it now seems clear that phosphorylations are uncoupled by DNP in at least some fully mature fruits (19). Pearson and Robertson (28) pointed out that, whereas an appropriate concentration of DNP accelerates the respiration of preclimacteric apple slices, no concentration of this reagent will produce this effect on climacteric tissue. This does not constitute evidence that DNP is without effect on phosphorylations in the mature apple. It rather indicates that the ATP/ADP ratio does not control the respiration rate at this stage in fruit development. The inhibition of ethylene production by DNP (fig 5) probably means that a phosphorylation is involved in the synthesis of ethylene. A similar result was reported earlier for Red Delicious apples (38), and has recently been observed in tomatoes (36).

Attempts were made to reverse the inhibition by supplying ATP and various other sources of energy. The high permeability of the cells (mentioned above) would be expected to allow the entry of a variety of molecular types. Glycerol alone at 0.55 M concentration caused a slight reversal of the inhibition of ethylene production without affecting respiration. This is no doubt attributable to a reversal of the effect of water soaking, and it is not directly concerned with the DNP inhibition. At a concentration as low as 5×10^{-2} M, compounds added in the absence of glycerol could not be expected to raise the rate of ethylene production above the 43 % value obtained when tissue was soaked in water alone. When this was found to

TABLE IV.

REVERSAL OF INHIBITION OF ETHYLENE PRODUCTION AND RESPIRATION CAUSED BY 2-4-DINITROPHENOL (DNP)

COMPOUNDS FED*	RATE AS % OF THAT IN GLYCEROL TREATED TISSUE**		
	C_2H_4 PRODUCTION	O_2 CONSUMPTION	CO_2 LIBERATION
Glycerol (G)	100	100	100
Water	43	100	100
DNP	12	33	40
DNP + G	25	36	42
DNP + G + ATP	50	59	52
DNP + G + *a*-keto glutarate	71	65	89
DNP + G + citrate	61	53	66
DNP + G + malate	80	80	125
DNP + G + acetyl-phosphate	54	60	57
DNP + G + pyruvate	53	47	93
DNP + G + oxaloacetate	50	39	61
DNP + G + fructose-diphosphate	83	69	80
DNP + G + glucose-6-phosphate	50	50	72
DNP + G + glucose-1-phosphate	74	50	72

* DNP applied at 5×10^{-5} M, glycerol at 0.55 M, and other compounds at 5×10^{-2} M. All solutions adjusted to pH 5.0. Additional compounds producing no effect: alanine, glycine, glycolamide, succinate, fumarate (slightly toxic), propionate, glucose, acetamide.

** Measurements made over a 60 minute period following the treatment.

FIG. 5–10. The rates of ethylene production △——△ and respiration ○——○ of tissue soaked in various concentrations of the inhibitors as compared to the rates of water soaked controls. All measurements were made over a 90 minute period after treatment.

FIG. 5 (*upper left*). Dinitrophenol.

FIG. 6 (*upper right*). Sodium fluoride.

FIG. 7 (*center left*). Iodoacetamide.

FIG. 8 (*center right*). Fluoroacetate.

FIG. 9 (*lower left*). Sodium arsenite. Upper curve O_2 consumption, lower curve C_2H_4 production.

FIG. 10 (*lower right*). Sodium bisulfite. The apparent rise in the respiration rate observed at high concentrations is almost certainly due to oxidative decomposition of the salt.

be true glycerol was included in all subsequent feedings to remove the restriction imposed by the water soaking. The rate observed in the presence of 0.55 M glycerol, 5×10^{-5} M DNP, and 5×10^{-2} M added compound was compared to the maximum rate which might be expected, namely that of tissue from the same apple treated with 0.55 M glycerol alone. The compounds were added in combination with DNP at pH 5.0, and the tissue was soaked in the solutions for 60 minutes at room temperature (table IV).

In general, if it is agreed that DNP does not interfere with substrate level phosphorylations (5, 16), then the results are consistent with the hypothesis that ATP or some equivalent high energy source is required for ethylene synthesis. The active compounds without exception can give rise to ATP or acetylcoenzyme A by known metabolic reactions. ATP may arise during glycolysis (hence from the sugar-phosphates) or coincident with the decarboxylation of *a*-keto glutarate (and citrate may give rise to *a*-keto glutarate indirectly). Acetylcoenzyme A is formed during oxidative decarboxylation of pyruvate (and pyruvate may be derived from malate and oxaloacetate) or from acetyl-phosphate directly. It is noteworthy that whenever ethylene production was partially reinstated by an added compound there was some coincident acceleration of respiration. With the possible exception of alanine, none of the inactive compounds (see footnote to table IV) could increase the ATP or acetylcoenzyme A level of the tissue by known pathways.

The special case of alanine was subjected to an additional experiment, since alanine might be converted via pyruvate to acetylcoenzyme A. A detectable increase in the carbon dioxide production of the tissue resulted when as little as 6×10^{-3} M sodium pyruvate was soaked into apple plugs, but the carbon dioxide production remained unchanged when even 100 times this concentration of alanine was applied. Apparently under the conditions of these experiments alanine is simply not converted to pyruvate in significant quantities.

Since fluoride lowers the respiration rate of the tissue, fluoride must decrease the availability of energy and thus produce somewhat the same overall effect as DNP. This may be the basis for the inhibitory effect of fluoride on ethylene production shown in figure 6. Attempts were made to reverse this inhibition in the same way as with DNP (summarized in table V). The stimulation with added ATP, and the lack of response to AMP, are certainly consistent with the idea that ATP or a high energy acylated compound is involved in ethylene synthesis. With minor exceptions the interpretations advanced for DNP can account for the observed results. An added consideration is that any stimulation of the electron transport system may now result in the production of energy. Taking this latter fact into account it is perhaps not surprising that the inhibitions caused by fluoride can be more completely reversed than those caused by DNP. While the activity of the sugar-phosphates is not easily reconciled with the known inhibitory action of fluoride on the enolase reaction, their stimulatory effect might be exerted through an alternative pathway of sugar metabolism.

TABLE V.

REVERSAL OF INHIBITION OF ETHYLENE PRODUCTION AND RESPIRATION CAUSED BY FLUORIDE

COMPOUNDS FED*	RATE AS % OF THAT IN GLYCEROL TREATED TISSUE**		
	C_2H_4 PRODUCTION	O_2 CONSUMPTION	CO_2 LIBERATION
Glycerol (G)	100	100	100
Water	42	100	100
NaF	25	52	50
NaF + G	55	64	58
NaF + G + pyruvate	110	75	92
NaF + G + acetyl-phosphate	84	87	64
NaF + G + ATP	100	84	79
NaF + G + citrate	82	83	78
NaF + G + malate	109	82	65
NaF + G + *a*-keto glutarate	75	62	63
NaF + G + oxaloacetate	70	53	58
NaF + G + fructose-di-phosphate	68	85	63
NaF + G + glucose-1-phosphate	70	91	68
NaF + G + glucose-6-phosphate	68	78	74
NaF + G + succinate	67	100	74
NaF + G + AMP	40	65	60

* NaF applied at 10^{-3} M, glycerol at 0.55 M, and other compounds at 5×10^{-2} M. All solutions adjusted to pH 5.0. Additional compounds producing no effect: alanine, glycine, glycolamide, hydracrylamide, glucose, fumarate and acetaldehyde (slightly toxic).

** Measurements made over a 60 minute period following the treatment.

Attempts to reverse the inhibition caused by iodoacetamide were unsuccessful (table VI). The failure of applied ATP and acetyl-phosphate to stimulate ethylene production indicates that more than energy is lacking in tissue treated with iodoacetamide. Since this inhibitor binds sulfhydryl groups generally, the inference is that a grouping of this type may be involved in one or more stages of ethylene synthesis.

DISCUSSION AND CONCLUSIONS

Evidence has been presented that the various substances which accelerate the rate of ethylene production in water-soaked apple sections exert their effect by a physical action. Since it appears that the solute concentration and not the water content of the cell is the critical factor, it may be concluded that the ultimate effect is being exerted on a body which is smaller than the whole cell and enclosed by an osmotic barrier. This body or particle must contain the ethylene producing system.

TABLE VI.

ATTEMPT AT REVERSAL OF INHIBITION OF RESPIRATION AND ETHYLENE PRODUCTION CAUSED BY IODOACETAMIDE

COMPOUNDS FED*	RATE AS % OF THAT IN GLYCEROL TREATED TISSUE**		
	C_2H_4 PRODUCTION	O_2 CONSUMPTION	CO_2 LIBERATION
Glycerol (G)	100	100	100
Water	51	100	100
Iodoacetamide (I)	19	54	56
I + G	52	56	63
I + G + pyruvate	50	51	83
I + G + acetyl-phosphate	51	56	45
I + G + ATP	49	58	59

* Iodoacetamide applied at 10^{-2} M, glycerol at 0.55 M and other compounds at 5×10^{-2} M. All solutions adjusted to pH 5.0.

** Measurements made over a one hour period following the treatment.

It is now well established that isolated mitochondria have osmotic properties, and that their gross appearance and biochemical activity may be dependent upon the tonicity of the solution in which they are suspended. Moreover, as demonstrated by Laties (17) and Opie (27), isolated mitochondria can stand dehydration but not swelling. The ethylene production of apple tissue behaves similarly, since it is inhibited by a lowered but not by an increased cellular solute concentration. Even plasmolyzed cells can still produce ethylene at maximal rate. Although most investigations of the effect of tonicity on mitochondria have been performed on isolated systems, adverse effects of water soaking on the mitochondria and metabolism of intact tissue have also been reported. Salt accumulation is controlled by the cytochrome-cytochrome oxidase system which is certainly associated with the mitochondria (32, 33). Stiles and Dent (37) found that prolonged washing could decrease salt accumulation and respiration in tuberous roots and stems. Laties (18) has shown that washing of cauliflower pieces lowers the amount of succinic dehydrogenase activity which could subsequently be isolated from the tissue, and succinic dehydrogenase is known to occur in the mitochondrial fraction (32). Elliot and Libet (8) observed that the respiration of brain minces suspended in water was considerably less than when the tissue was in an isotonic environment. However, if the ethylene producing system is in a particle, there would be no reason a priori to suppose that the system is in the same particle as that which contains the respiratory enzymes. In fact, there is evidence which at first sight appears to the contrary. Smock and Sparrow (35) for instance, reported that apples irradiated with 20,000 to 40,000 r of gamma radiation showed a reduced rate of ethylene production but an unchanged respiration rate. Hansen (15) found that at high temperatures the ethylene production of pears was severely retarded, but the respiration stimulated. Thus irradiation and high temperatures produce effects similar to water soaking, which, as described in this paper, reduces the ethylene production of tissue sections but does not affect the respiration rate. As against this evidence of separation between the two functions, two other facts seem to establish a close relationship between respiration and ethylene formation. In the first place, the threshold concentration of all inhibitors was found to be the same for both processes. Moreover, throughout a large concentration range of each of several poisons, the inhibition of ethylene production was closely comparable to that of respiration (figs 5 through 10). Secondly, the response to oxygen tension of the two processes is nearly identical; both reached half their maximum rate at about 1.5 to 2.0 % O_2 (4). As a working hypothesis, therefore, it is proposed 1) that the system producing ethylene is located in a particle; 2) that it is dislocated by even a slight swelling of the structure; 3) that it is very closely linked, at least for an oxygen consuming step (4), with the terminal oxidase, and 4) that it is dependent upon the respiratory process for energy. The different responses of the respiratory and ethylene producing systems to osmotic and other physical changes might be accounted for in one of two ways. Both systems may be located in the same particle, presumably a mitochondrion, but the enzymes functioning in ethylene synthesis might be much more susceptible to inactivation. Alternatively, and perhaps less probably, two distinct particles may be involved.

It is interesting that the concentration of glycerol (ca 0.23 *N* (fig 4)) which just maintains the tissue at its initial water content, is the lowest concentration which suffices to produce a measurable effect on the ethylene production (see fig 1). A 100 % response, however, is not obtained until a somewhat higher glycerol concentration is applied. This suggests that glycerol is not as efficient in maintaining the ethylene producing particles as is the normal solute mixture present in the cells. There is, indeed, other evidence for some sort of specificity. For instance, although 1,2-propane diol is closely related to glycerol and would be expected to penetrate into the tissue more easily than glycerol does, even very high molarities of 1,2-propane diol did not produce the same effect as glycerol. Similarly, certain of the other organic substances were not as active as might be expected in terms of the rate at which they probably entered into the tissue. What must be considered, however, is not only the permeability of the plasma membrane but also the permeability of the particle membrane. A substance which enters the cell very easily might also pass into the particle and thus would not be able to affect the turgor of that body. The steepness of the curves shown in figure 1 suggests that the particle is extremely sensitive to a very slight decrease in the osmotic content of its environment, for raising the normality of the applied solutions from 0.2 to 0.4 *N* covers the entire range from zero

to 100 % effect. With a system of such sensitivity it is conceivable that very slight differences in the permeability of the applied compounds might account for the apparent specificity.

It might be expected that the absolute value of the cellular osmotic concentration would have no bearing on the activity of the enzymes contained in a particle as long as the contents of the particle were in complete equilibrium with their environment. Otherwise, marked metabolic changes might be expected to result from slight variations in the osmotic content of a cell. However, evidence does exist that in the case of ethylene production the osmotic conditions under which cells are growing may be crucial. Two groups have studied the effect of varied carbon sources on the production of ethylene by *Penicillium digitatum*. Fergus (9) reported that glucose did not support ethylene production as well as did sucrose. Phan-Chon-Ton (30) found that glycerol and alanine greatly increased the production of ethylene, whereas acetaldehyde and pyruvate also produced small increases. He concluded that glucose was a better carbon source than sucrose, whereas fructose was inferior to sucrose. Ulrich (personal communication) noted that glycine accelerated the production of ethylene. With the exception of acetaldehyde, which is toxic to both respiration and ethylene production in apple tissue, all of the compounds which accelerated the ethylene production of *Penicillium digitatum* are also among those which prevent the inhibition caused by soaking apple sections in water. If the biochemistry of ethylene production is the same in this fungus as in the apple it would follow that the observed acceleration of ethylene production in *Penicillium digitatum* is not due to the provision of substrate. Indeed, Phan-Chôn-Ton's data (30) show that, with the exception of fructose, there is a direct correlation between the osmotic concentration of the medium and its ability to support ethylene production. These experiments with *Penicillium digitatum* strongly suggest that the formation of the enzyme system which produces ethylene was favored by raising the solute concentration of the medium.

Calculations based on values for the standard free energies of formation of ethylene and many common biological intermediates show that there is in all cases a net loss of free energy in the conversion of unit activities of these intermediates to ethylene (2). However, in all hypothetical schemes, one step, the introduction of the double bond, involves an increase in free energy. Evidence has been presented earlier (2, 4) that one of the terminal steps in the formation of ethylene is a dehydration; it seems probable that this may be the reaction in which the double bond is formed. If, as the inhibitor studies indicate, respiration provides energy for the synthesis of ethylene, then this energy may be utilized in or just prior to the terminal reaction in which the molecule is desaturated. Moreover, it should not be possible to inhibit tissue respiration without simultaneously inhibiting the ethylene production. Three instances of the converse effect, in which ethylene production is decreased or eliminated without a concomitant decline in respiration, were mentioned above. On the other hand, there does not seem to be a single clear-cut example in which respiration can be inhibited without a similar effect on ethylene production. Such evidence as there is, therefore, favors this interpretation.

Summary

The ethylene production of McIntosh apple tissue sections was studied by means of a highly sensitive gas chromatographic technique.

Apple sections show a wound response which consists of an accelerated respiratory rate and a decreased intensity of ethylene production. Soaking the tissue in water for 60 minutes depressed the rate of ethylene production by more than 50 %; this soaking did not affect the respiration rate of the tissue.

The inhibition of ethylene production caused by soaking in water was completely prevented by high concentrations of glycerol, hydracrylic amide or potassium chloride, and partially prevented by a large variety of other compounds. In no case, however, could the rate of ethylene production be raised above that of unsoaked controls. Correspondingly, evidence is presented that the various substances do not act at the substrate level, but rather regulate the solute content of the cell. It is deduced that the ethylene producing mechanism is located in a particle that is extremely sensitive to the solute concentration of the cell sap, the particle being able to withstand dehydration but not swelling.

The water relations of apple tissue were found to be atypical: the cells lost relatively large amounts of water and solute when suspended in water, whereas they actually gained water when suspended in high molarity glycerol solutions.

The effects of several inhibitors on ethylene production and respiration were investigated. DNP, fluoride, iodoacetamide, arsenite, sodium bisulfite and fluoroacetate all inhibited both processes to about the same extent over wide concentration ranges and in each case with equivalent thresholds. The inhibitions caused by DNP, and to an even greater extent those caused by fluoride, were partially reversed by ATP or other energy sources; this suggests that at least one step in the synthesis of ethylene requires energy which is supplied by respiration. Another step may involve a sulfhydryl enzyme, since high energy compounds failed to reverse the inhibitory effect of iodoacetamide. The lack of inhibition with even high concentrations of cyanide makes it unlikely that an iron containing enzyme is involved in ethylene synthesis, whereas the results with bisulfite suggest that none of the immediate precursors is an aldehyde susceptible to bisulfite addition. The close relationship between ethylene formation and respiration, as well as previous work on the oxygen sensitivity curves for both processes, indicate that ethylene may be produced in the mitochondria.

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