**Ether Wash Solution.** The addition of the small amount of water to the anhydrous reagent grade ether had no effect upon the results from synthetic betaine standards. This was not so when water was added to U.S.P. ether. Apparently the small amount of alcohol present in the U.S.P. ether combines with the water to dissolve some of the betaine reineckate. The wash solution of anhydrous ether with a small amount of water was chosen because it was somewhat easier to transfer the crystals and displace the mother liquor with this solution.

**Stability of Betaine Reineckate Color.** The data in Table I show that 10 to 14% of the betaine reineckate color in acetone is lost over an 18-hour period. Therefore, it is advisable to measure the absorbance of the samples within 2 hours after the betaine reineckate has been dissolved in 70% acetone.

**Effect of Sample Size and Recovery of Added Betaine.** Experiments on sample size and recovery were carried out on an end liquor sample (Tables II and III). The results illustrate that a linear relationship exists between the sample size and color being measured over the range of sample concentrations tested, and that satisfactory recovery of known added amounts of betaine can be obtained. Therefore, it can be concluded that other compounds present in the original material have very little if any effect upon the results. The absence of an insoluble reineckate precipitate in an alkaline solution of these samples and a negative test for amino acids on the precipitate by paper chromatography substantiate this conclusion.

**Analysis of Process Samples.** The results of duplicate analyses on several process samples are given in Table IV. These samples were analyzed on the same day by one analyst and a standard curve was prepared at the same time. Therefore, they illustrate the degree of precision that can be obtained by the method. It can be concluded that the method will give reliable and consistent results on the process samples tested.

### Discussion

As the exact composition of the betaine reineckate precipitate is not known, the method is empirical. Therefore, it is necessary to prepare a standard curve under the same conditions used for unknowns.

The method is not specific, since many organic bases of high molecular weight are precipitated by Reinecke salt in acid solution (*2*, *3*, *8*). It is particularly applicable to samples containing a considerable amount of betaine, where interferences can be avoided by diluting. However, as shown in the work on mixtures of betaine and choline (*1*), many interferences can be avoided by a preliminary precipitation of reineckates insoluble in alkaline solution.

The method is especially suited for routine control, as it is simple and capable of yielding results with a fair degree of precision. With proper regard to standardization and interferences it should find considerable use as a method for betaine in other material from natural sources.

### Literature Cited


(1) Bandelin, F. T., Pankratz, R. E., *J. Am. Pharm. Assoc.* **42,** 442 (1953).
(2) Glick, D., *J. Biol. Chem.* **156,** 643–51 (1944).
(3) Kahane, E., *J. pharm. chim.* **22,** 254–67 (1935).
(4) Parnöja, E., *Socker* **8,** 75–7 (1954).
(5) Stanek, V., *Z. physiol. Chem.* **47,** 83 (1904).
(6) Strack, E., Schwaneberg, H., *Ibid.*, **245,** 11–18 (1936).
(7) Walker, H. G., Jr., Erlandsen, R., *Anal. Chem.* **23,** 1309–11 (1951).
(8) Wilson, J. B., *J. Assoc. Offic. Agr. Chemists* **35,** 455–8 (1952).



*Received for review July 18, 1955. Accepted February 20, 1956.*


**FROZEN FOODS STORAGE EFFECTS**

# Formation of Alcohol, Acetaldehyde, and Acetoin in Frozen Broccoli Tissue


**P. A. BUCK[1] and M. A. JOSLYN**
**University of California, Berkeley, Calif.**



The carboxylase activity of broccoli tissues was investigated to determine the role of pyruvic carboxylase in the production of volatile aldehydic and ketonic compounds which might serve as precursors of the off-flavors developing in underscalded frozen broccoli. Broccoli carboxylase was found to catalyze synthesis of acetoin and diacetyl chiefly from added pyruvate and to a much smaller extent from acetaldehyde, unlike pea and wheat germ carboxylase. Acetaldehyde inhibited broccoli carboxylase activity. In frozen broccoli, both inhibition by and restricted diffusion of acetaldehyde would favor production of acetoin. The concentration of acetaldehyde, acetoin, or diacetyl was not related to the organoleptically objectionable formation of off-flavors, whereas ethyl alcohol content was related to extent of off-flavor.


INVESTIGATIONS on the accumulation of acetaldehyde, ethyl alcohol, and related products in broccoli during freezing storage (*3*) were continued, with particular emphasis on pyruvic carboxylase activity. These investigations of the nature of the biochemical reactions which result in abnormal flavors in improperly scalded, frozen vegetables also led to a study of the behavior of pyruvic carboxylase in situ. The immature floral shoot of sprouting broccoli was used as the source of the enzyme system. Additional data on the accumulation of ethyl alcohol and acetoin are reported here and in more detail in the thesis of Buck (*2*).

[1] Present address, Department of Dairy and Food Industries, University of Wisconsin, Madison, Wis.

### Materials and Methods

Commercially grown Italian green sprouting broccoli (whose structure is shown in Figure 1), packed in crates with ice, was obtained from a local wholesale distributor and stored at 1° C. The broccoli was obtained in several lots in order to ensure fresh tissues at the time of utilization. Broccoli shoots were

washed in water, dried by draining, and chilled or frozen before homogenization. Sterilization of the surface of broccoli floral shoots with chemical disinfectants required vigorous agitation for at least 20 minutes, followed by several rinsings with sterile, distilled water. This treatment altered enzymatic activity. Control samples agitated with distilled water had increased gas exchange due to the bruising; and samples soaked in a solution of disinfectant without agitation were contaminated sporadically, hence were unreliable. Solutions of 1 to 3000 Roccal (alkyl dimethyl benzyl ammonium chloride compound supplied by Winthrop Chemical Co.), 7% calcium hypochlorite, or 1% mercuric chloride disinfectants reduced the enzyme activity slightly, whereas less concentrated solutions which did not affect the activity were ineffectual. Therefore, only the anaerobic experiments were performed using antiseptic technique. The regular experiments were performed using broccoli which had been thoroughly washed in flowing, cold tap water and rinsed in flowing distilled water.

After the broccoli had been washed thoroughly, it was drained in clean cheesecloth and chilled by storing the intact floral shoot at 1° C. for several hours. Sometimes the tissues were frozen at −17° C. in order to facilitate their maceration or to test the effect of freezing. The floral shoots were then either cut into the desired tissue sections and organs using a stainless steel knife, or ground to a uniform mixture in a Universal food chopper using the fine blade. The food chopper and all equipment for preparation of samples were stored continuously at 1° C. to maintain a uniformly chilled environment throughout the preparation. (Preliminary investigations showed that the broccoli floral shoot must be extracted at low temperature to prevent denaturation of carboxylase.) After the shoot had been sectioned or ground, the material was mixed and aliquots were taken for analysis. The remainder of the material was stored in a glass-stoppered container at 1° C. for not exceeding 3 days, or at −17° C. for 1 day to several months. Control samples which were not macerated were stored simultaneously.

In addition, samples of broccoli were available which had been scalded for varying lengths of time and stored at −17° C. for 7 years. The preparation of these samples has been reported (*3*).

A partially purified fairly stable carboxylase preparation was obtained by macerating cut broccoli in a Waring Blendor with an equal amount of added water at 1° C., and centrifuging the resulting mixture first at 1000 × gravity to remove coarse particles and then at 16,000 × gravity to remove more finely suspended plastid and mitochondrial particles. This preparation was used in investigations on acetaldehyde and acetoin formation from added pyruvate. Yeast carboxylase (*7*, *9*) and wheat germ carboxylase (*10*) have been shown to possess both decarboxylating and acetoin synthesizing activities.

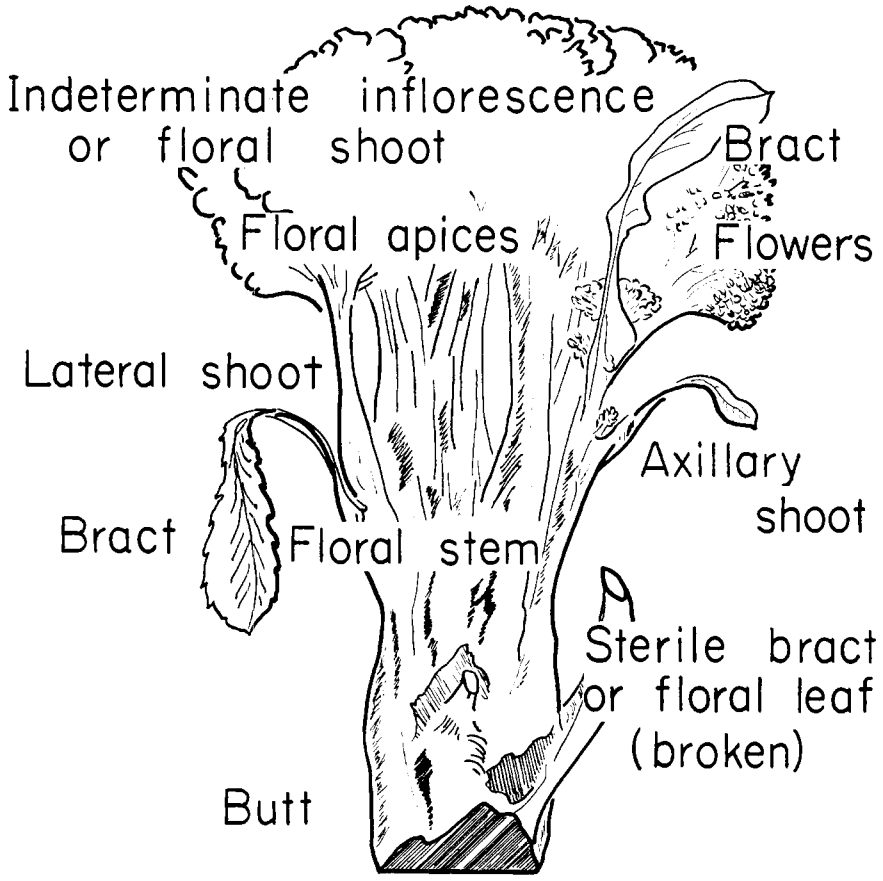


Figure 1. Morphology of the floral shoot of broccoli

**Analytical Methods.** The volatile aldehyde, ethyl alcohol, total solids, and ascorbic acid content were determined by methods used previously. Total nitrogen was determined by the micro-Kjeldahl distillation method of the Association of Official Agricultural Chemists (*1*). Acetoin was determined qualitatively using the modified Barritt reagent of Vaughn, Wedding, and Tabachnick (*13*), which was mixed just before use. A sensitive test was obtained when the 1-naphthol was resublimed under vacuum (*5*). Acetoin and diacetyl were determined quantitatively on the steam distillate collected for acetaldehyde determination by the colorimetric method of White, Krampitz and Werkman (*15*). Acetoin was oxidized before its determination by the modified Werkman method (*11*), and the amount of acetoin was the difference between the calculated amounts of diacetyl in the oxidized and unoxidized samples. For the micro amounts of acetoin, an efficient fractionating column was employed. Micrograms of diacetyl were read from a calibration curve for pure diacetyl, using a Klett-Summerson photoelectric colorimeter with a 420 mμ filter; 100 γ of of diacetyl gave a colorimetric value of 130.

## Results

**Acetaldehyde and Acetoin Formation by Broccoli Carboxylase Preparation.** During decarboxylation of pyruvic acid, acetaldehyde was the predominant product, but in certain experiments increased amounts of acetoin were formed. The accumulation of acetoin by different enzyme preparations, as well as the rate of evolution of carbon dioxide, varied with the method of extraction. A possible shift in the catalysis of carboxylase from straight decarboxylation of pyruvic acid to decarboxylation and condensation of the product was indicated.

Changes in the amount of acetoin formed often did not parallel changes in the rate of carbon dioxide evolved, despite precautions that were taken to avoid errors of precedure. The cause of this phenomenon was sought by evaluating the modifications in the method of extraction. The addition of thiamine did not alter the ratio of acetoin to carbon dioxide. When added during extraction, thiamine increased not only the

**Table I. Effect of Ratio of Thiamine Pyrophosphate to Pyruvic Acid on Synthesis of Acetoin by Broccoli Carboxylase**

| Pyruvate Molarity | Thiamine Pyrophosphate Molarity × $10^5$ | Ratio of Cocarboxylase / Pyruvic Acid × $10^2$ | $Q_{CO_2}$[a] | Acetoin[b] |
|---|---|---|---|---|
| 0.0015 | 6.4 | 4 | 121 | 570 |
| | 33 | 22 | 156 | 80 |
| 0.003 | 6.4 | 2 | 152 | 609 |
| | 33 | 11 | 183 | 166 |
| 0.006 | 6.4 | 1 | ... | 712 |
| | 33 | 5.5 | 221 | 650 |
| 0.009 | 6.4 | 0.7 | 168 | 520 |
| | 33 | 3.6 | 243 | 656 |
| 0.015 | 6.4 | 0.4 | 182 | 300 |
| | 33 | 2.2 | 256 | 736 |
| 0.03 | 6.4 | 0.2 | ... | 106 |
| | 33 | 1.1 | 283 | 361 |
| 0.045 | 6.4 | 0.14 | 222 | 30 |
| | 33 | 0.7 | 283 | 289 |
| 0.060 | 6.4 | 0.1 | 234 | 0 |
| | 33 | 0.55 | 288 | 266 |
| 0.090 | 6.4 | 0.07 | 234 | 0 |
| | 33 | 0.36 | 422 | 259 |
| 0.15 | 6.4 | 0.04 | ... | 0 |
| | 33 | 0.22 | 369 | 170 |
| 0.3 | 6.4 | 0.02 | ... | ... |
| | 33 | 0.11 | ... | 74 |

[a] Microliters of $CO_2$ liberated per ml. of tissue extract per hour.
[b] Relative concentration measured as Klett colorimeter reading.

carboxylase activity, but also the synthesis of acetoin.

The partially purified preparation of carboxylase used for the study of its carboligase activity was obtained by a modification of the procedure described initially. Five hundred grams of broccoli were ground in a food chopper at 0° C. and the macerate was squeezed in a Carver press at 5000 to 10,000 pounds per square inch at 0° C. The exudate was collected in 100 ml. of chilled cysteine-thiamine solution containing 0.01*M* of each reagent. After centrifuging at 2000 × gravity to remove coarse particles, the solution was centrifuged at 16,000 × gravity for 20 minutes as before. To this supernate, neutralized ammonium sulfate was added slowly during stirring until the solution was half saturated. The solution was centrifuged and decanted, and the precipitate was dialyzed in distilled water at 1° C. for 6 hours. Further details of this enzyme preparation will be published (*8*).

With carboxylase isolated as described, addition of thiamine decreased carboxylase activity and acetoin formation. The carboxylase activity and synthesis of acetoin were not affected by the addition of magnesium ion to extracts. The atmosphere during analysis did not influence the formation of acetoin. The amount of acetoin formed during decarboxylation under nitrogen, when carbon dioxide accumulated, was similar to the amount formed in the presence of alkali, when no carbon dioxide accumulated. Likewise, the amount of acetoin formed in nitrogen and in air was similar, though the concentration of oxygen differed.

The relative concentration of pyruvic acid and thiamine pyrophosphate in the reaction mixture markedly affected the extent of acetoin formation, as shown in Table I. Increased amounts of pyruvic acid, added to the enzyme preparation containing $6 \times 10^{-4}M$ thiamine pyrophosphate, inhibited the formation of acetoin, while the evolution of carbon dioxide increased to a maximum. Magnesium ion, buffer, and enzyme were added. Although the amount of carbon dioxide evolved from the lowest concentration of pyruvic acid totaled only about 90 $\mu$l., the rate within the first 20 minutes was uniform, so that the amount has been expressed in the table in terms of milliliters of $Q_{CO_2}$ for comparison with other values. The amount of acetoin has been expressed as a relative value determined by measurement of absorption of light by the colored solution obtained from the qualitative test for acetoin. The absorption of light was measured in a Klett-Summerson photoelectric colorimeter with a 540-m$\mu$ green filter.

Two levels of thiamine pyrophosphate were used with varied levels of pyruvic acid. Maximum evolution of carbon dioxide was dependent upon the concentration of pyruvate and occurred in these experiments at 0.09*M* pyruvic acid. The greatest synthesis of acetoin, however, occurred at thiamine pyrophosphate–pyruvic acid ratios ranging from $2 \times 10^{-2}$ to $1 \times 10^{-2}$. Aliquots obtained by combining duplicate manometric samples were analyzed quantitatively for acetoin and diacetyl. The sample that gave the maximum amount of acetoin, determined by light absorption, contained 37 $\gamma$ per ml. This amount of acetoin formed was 1.1% of the added pyruvic acid. Diacetyl was detected only in trace amounts.

**Acetaldehyde, Ethyl Alcohol, Acetoin, and Diacetyl Formation in Broccoli Tissues.** The formation of compounds originating from the decarboxylation of pyruvic acid by broccoli tissues was investigated. Differential determinations of acetoin and diacetyl were performed on samples of tissues stored anaerobically at room temperature, and on samples stored at −17° C.

Analysis of Anaerobic Samples. Because reaction rates are slow at low temperatures, samples of tissue with added pyruvic acid and acetaldehyde were placed under anaerobic conditions at room temperature for 4 hours in order to investigate the effect of pyruvic acid and acetaldehyde on the formation of acetoin. Control samples were placed under similar anaerobic conditions for varying periods of time.

Broccoli was cleaned, so that the contribution from microbial contamination was insignificant when the broccoli was held at room temperature under nitrogen. The counts obtained by the plate method ranged from 10 to $10^2$ microorganisms per gram of fresh tissue. Two hundred grams of broccoli shoots were shaken for 20 minutes with 200 ml. of 7% calcium hypochlorite solution. The sample then was aseptically drained, rinsed three times with 100-ml. portions of sterile, distilled water, cooled by freezing at −17° C., and finally ground in a Universal food chopper at 1° C. Twenty-five grams of the pulp were placed in sterilized, glass-stoppered flasks. Pyruvic acid was added to duplicate preparations of tissues to produce $3.6 \times 10^{-2}M$ and $1.8 \times 10^{-2}M$ concentrations. To similar preparations, acetaldehyde was added in final concentrations of $35 \times 10^{-2}M$ and $7 \times 10^{-2}M$. A mixture containing $1.8 \times 10^{-2}$ and $7 \times 10^{-2}M$ acetaldehyde was added to still another preparation. Control samples were prepared simultaneously. All samples were flushed with nitrogen at a rate of 800 ml. per minute for 15 minutes. The flasks were hermetically sealed, and the samples were held for 4 hours at room temperature (about 23° C.), and then were frozen prior to analysis.

In another experiment, samples of 100 grams of broccoli shoots were prepared similarly, but the tissues were not ground and no substrates were added. The air was removed from the tissues by evacuating the flasks using a Nelson pump, and nitrogen was introduced into the flasks before they were sealed. These samples were held for varying lengths of time at room temperature before being frozen and ground for analysis.

The flasks were opened and quickly connected to glass steam distillation equipment, and 250 ml. of distillate were collected for analysis. The results of the analyses are given in Table II. Even under anaerobic conditions, diacetyl accumulated in all shoots, whereas acetoin accumulated in the macerated tissue. The addition of pyruvic acid to the pulp caused accumulation of ethyl alcohol, acetoin, and diacetyl. In the tissues to which acetaldehyde was added, 99% of the lower level and 67% of the higher level of acetaldehyde were recovered when the products were included. Ethyl alcohol accumulated in large amounts, but acetoin accumulated only slightly. The acetoin and diacetyl synthesis was suppressed compared to the pyruvate series. In macerated tissue to which both substrates were added, ethyl alcohol and diacetyl accumulated, but no acetoin was detected.

Analysis of Frozen Broccoli Samples. Frozen samples of raw and scalded broccoli shoots which had been stored at −17° C. for 7 years were analyzed to determine if acetaldehyde, ethyl alcohol, acetoin, and diacetyl had accumulated. The tissues initially had been analyzed for acetaldehyde and ethyl alcohol, but not for acetoin or diacetyl (*3*). The tissues which had been subjected to the most severe scalding did not accumulate acetaldehyde or ethyl alcohol and were regarded as controls. Because any initial amount of acetoin might have decreased in these tissues during the scalding process, several samples of fresh broccoli were also analyzed. Duplicate samples were frozen and stored for various periods of time up to 1 year.

The analyses are given in Table III. Acetaldehyde did not accumulate significantly, if at all, during storage at −17° C. for 1 year. The increase in the amount of acetaldehyde in the raw sample stored for 2 months was atypical, and might have occurred before the packaged broccoli became frozen. A sample held aerobically for 1 month at 0° C. accumulated 3.97 mg. of acetaldehyde and only 26.6 mg. of ethyl alcohol per 100 grams of tissue.

During 7 years of storage at −17° C., raw broccoli accumulated acetaldehyde and ethyl alcohol. Samples which had been scalded for less than 3 minutes accumulated diacetyl. Acetoin was not detected in any sample of frozen broccoli shoots, either initially or after storage for 7 years.

**Table II. Acetaldehyde, Ethyl Alcohol, Acetoin, and Diacetyl Formation during Anaerobiosis of Broccoli Pulp and Broccoli Shoots**

(Pyruvic acid and acetaldehyde added to some tissue preparations)

| Sample | Holding Time, Hours | Substrate and Molarity × $10^2$ | Products, Mg./100 G. Broccoli Tissue: Acetaldehyde | Ethyl alcohol | Acetoin | Diacetyl |
|---|---|---|---|---|---|---|
| Pulp | 4 | 1.8 pyruvate | 4.0 | 123.8 | 1.9 | 2.0 |
| | | 3.6 pyruvate | 5.1 | 134.0 | 1.7 | 2.5 |
| | | 7 acetaldehyde | 15.8 | 288 | 0.3 | 0.1 |
| | | 35 acetaldehyde | 29.6 | 1010 | 0.3 | 0.45 |
| | | 1.8 pyruvate + 7 acetaldehyde | 14.5 | 323 | 0 | 2.0 |
| | | Control O | 3.9 | 33.4 | 1.6 | 0.06 |
| Shoots | 0 | 0 | 1.4 | 27.8 | 0 | 0.07 |
| | 4 | 0 | 3.0 | 30.4 | 0 | 0.71 |
| | 12 | 0 | 3.6 | 63.8 | 0 | 1.07 |
| | 23 | 0 | 4.7 | 96.0 | 0 | 1.48 |
| | 39 | 0 | 3.4 | 159.6 | 0.7 | 1.43 |

## Discussion

**Accumulation of Acetaldehyde and Ethyl Alcohol.** Alcohol dehydrogenase has been reported to occur in many plants (*6*, *12*), but the acetaldehyde–ethyl alcohol system in broccoli has not been previously studied. Broccoli tissues have been shown to contain pyruvic carboxylase (cf. *14*). Alcohol dehydrogenase also was assumed to be present, since ethyl alcohol served as a substrate for an enzymatic reaction which was poisoned by $10^{-4}M$ iodoacetate. When broccoli tissues were stored at 0° C., acetaldehyde accumulated, while ethyl alcohol increased only slightly. When the tissues were stored at −17° C., or were stored anaerobically, ethyl alcohol accumulated. The ratio of ethyl alcohol to acetaldehyde varied as follows:

| | Ethyl Alcohol / Acetaldehyde |
|---|---|
| Tissues stored 1 month at 0° C. | 6.7 |
| Tissues stored 1 year at −17° C. | 28 |
| Tissues stored 7 years at −17° C. | 58 |
| Tissues stored anaerobically up to 24 hours | 20 |
| Tissues stored anaerobically up to 40 hours | 47 |
| Fresh tissues, average | 10 |

In frozen tissues, acetaldehyde accumulated slightly, but ethyl alcohol accumulated markedly, as it did in anaerobic tissues. The ratios of ethyl alcohol to acetaldehyde in frozen tissues were typical of ratios of anaerobic tissues, and

**Table III. Acetaldehyde, Ethyl Alcohol, Acetoin, and Diacetyl Formation during Storage of Frozen Broccoli Shoots**

| Scalding Processes at 100° C., Minutes | Storage Period at −17° C. | Products, Mg./1000 G. Broccoli Tissue: Acetaldehyde | Ethyl alcohol | Acetoin | Diacetyl |
|---|---|---|---|---|---|
| 0 | 0 | 0.56 | 31.1 | 0 | 0 |
| 0 | 2 months | 1.40 | 30.6 | 0 | 0.072 |
| 1 | 2 months | 0.87 | 27.1 | 0 | 0 |
| 1 | 2 months | 0.67 | 12.0 | 0 | 0 |
| 4 | 2 months | 0.45 | 9.8 | 0 | 0 |
| 0 | 6 months | 0.97 | 18.2 | 0 | 0 |
| 0 | 1 year | 0.93 | 26.3 | 0 | 0 |
| 0 | 7 years | 2.30 | 133 | 0 | 0·42 |
| 1 | 7 years | 1.43 | 37.3 | 0 | 0.25 |
| 2 | 7 years | 0.96 | 24.2 | 0 | 0.251 |
| 3 | 7 years | 0.75 | 15.7 | 0 | 0.03 |
| 4 | 7 years | 0.77 | 14.2 | 0 | 0 |

indicated that in frozen tissues pyruvic acid was metabolized anaerobically, and most of the acetaldehyde was converted to ethyl alcohol.

**Accumulation of Acetoin and Diacetyl.** The formation of acetoin has been reported in the investigations of yeast and wheat germ carboxylase, but diacetyl seldom has been determined separately. David and Joslyn (*4*) analyzed a slurry of peas after 16 hours of storage at room temperature and found that at $2 \times 10^{-2}M$ pyruvate, large amounts of diacetyl and little acetoin were formed. However, the pea slurry was shaken for 16 hours in air, and spontaneous oxidation of acetoin could have occurred. They also found that at $5 \times 10^{-2}M$ acetaldehyde, only half the amount of acetoin and diacetyl were formed in equal amounts. When both $2.5 \times 10^{-2}M$ acetaldehyde and $10^{-2}M$ pyruvate were added, the amount of products at the end of 16 hours was the same as for acetaldehyde, but diacetyl was in greater concentration. Singer and Pensky (*10*), using purified wheat carboxylase, found that addition of pyruvate always increased the rate of acetoin formation to four times that obtained from acetaldehyde alone. When both acetaldehyde and pyruvate were present, maximal velocity was reached at approximately $5 \times 10^{-3}M$ pyruvate and $5 \times 10^{-2}M$ acetaldehyde. When acetaldehyde was the substrate, saturation was reached at $2 \times 10^{-1}M$ acetaldehyde, and half-saturation at $2 \times 10^{-2}M$. When pyruvate was the substrate at a concentration of $5 \times 10^{-3}M$, Singer and Pensky found that the amount of acetoin synthesized was only 5% of the amount formed in the presence of excess acetaldehyde. They state that when the pyruvate concentration was raised to $5 \times 10^{-2}M$, a significant amount of acetoin formed, although never as much as with acetaldehyde in excess.

In this study, samples of frozen broccoli were found to contain diacetyl, but little acetoin. Oxidation of acetoin to diacetyl might have occurred, even though frozen tissues were sufficiently anaerobic to permit ethyl alcohol to accumulate. In broccoli tissue to which pyruvic acid was added at $1.8 \times 10^{-2}M$, acetoin and diacetyl both accumulated (more diacetyl than acetoin). The addition of acetaldehyde resulted in the formation on only one twentieth of the amount of acetoin and diacetyl formed from pyruvic acid. Acetaldehyde was added in the same excessive amount as was added by Singer and Pensky. When acetaldehyde was added with pyruvic acid, the same amount of diacetyl was formed, but acetoin was not detected. The relative amounts of acetoin and diacetyl formed in these three plant tissues are shown in Table IV.

Isolated broccoli carboxylase, in the presence of thiamine pyrophosphate, synthesized acetoin from pyruvate, and in anaerobic tissues pyruvate caused extensive synthesis of acetoin and diacetyl. Carboxylase undoubtedly was involved in their synthesis in both anaerobic and frozen tissues.

Although diacetyl contributes to the flavors of many types of food organoleptically, the flavor change in broccoli did not seem to be due to the increase of diacetyl. The amounts of acetaldehyde, acetoin, and diacetyl in the tissues of the floral shoots could not be correlated with the intensity of the undesirable flavor.

**Table IV. Relative Amounts of Acetoin and Diacetyl Formed in Plant Tissues from Added Substrates**

| Preparation | Added Substrate | Relative Ratio | | |
|---|---|---|---|---|
| | | Acetoin | Diacetyl | Acetoin + diacetyl |
| Pea slurry | $2 \times 10^{-2}M$ pyruvate | 1 | 940 | 2 |
| | $5 \times 10^{-2}M$ acetaldehyde | 16 | 27 | 1 |
| | $1 \times 10^{-2}M$ pyruvate + $2.5 \times 10^{-2}M$ acetaldehyde | 5 | 39 | 1 |
| Wheat carboxylase | $5 \times 10^{-3}M$ pyruvate | .. | ... | 1 |
| | $5 \times 10^{-2}M$ pyruvate | .. | ... | 20 |
| | $2 \times 10^{-1}M$ acetaldehyde | .. | ... | 20 |
| | $5 \times 10^{-3}M$ pyruvate + $5 \times 10^{-2}M$ acetaldehyde | .. | ... | 40–80 |
| Broccoli pulp | $1.8 \times 10^{-2}M$ pyruvate | 19 | 20 | 10 |
| | $3.6 \times 10^{-2}M$ pyruvate | 17 | 25 | 10 |
| | $7 \times 10^{-2}M$ acetaldehyde | 3 | 1 | 1 |
| | $3.5 \times 10^{-1}M$ acetaldehyde | 3 | 5 | 2 |
| | $1.8 \times 10^{-2}M$ pyruvate + $7 \times 10^{-2}M$ acetaldehyde | 0 | 20 | 5 |

## Literature Cited


(1) Assoc. Offic. Agr. Chemists, Washington, D. C., "Official Methods of Analysis," 7th ed., 1950.
(2) Buck, P. A., Ph.D. thesis, University of California, 1954.
(3) Buck, P. A., Joslyn, M. A., J. Agr. Food Chem. **1,** 309 (1953).
(4) David, J. J., Joslyn, M. A., *Food Research* **18,** 390 (1953).
(5) Gattermann, L., Wieland, H., "Laboratory Methods of Organic Chemistry," Macmillan, New York, 1937.
(6) Goddard, D. R., Stafford, H. A., *Ann. Rev. Plant Physiol.* **5,** 115 (1954).
(7) Green, D. E., Herbert, D., Subrahmanyan, V., *J. Biol. Chem.* **138,** 327 (1941).
(8) Joslyn, M. A., Buck, P. A., unpublished manuscript.
(9) Juni, E., *J. Biol. Chem.* **195,** 727 (1952).
(10) Singer, T. P., Pensky, J., *Biochim. Biophys. Acta* **9,** 316 (1952); *J. Biol. Chem.* **196,** 375 (1952).
(11) Stotz, E., Roborg, J., *Ibid.*, **150,** 25 (1943).
(12) Stumpf, P. K., *Ann. Rev. Plant Physiol.* **3,** 17 (1952).
(13) Vaughn, R. H., Wedding, G. T., Tabachnick, J., *Hilgardia* **18,** 357 (1948).
(14) Vennesland, B., Felsher, R. Z., *Arch. Biochem.* **11,** 279 (1946).
(15) White, A. G. C., Krampitz, L. O., Werkman, C. H., *Arch. Biochem.* **9,** 229 (1946).


*Received for review July 25, 1955. Accepted February 10, 1956.*