CXXXVIII. THE CONTROLLING INFLUENCE OF CARBON DIOXIDE.

V. A QUANTITATIVE STUDY OF THE PRODUCTION OF ETHYL ALCOHOL AND ACETALDEHYDE BY CELLS OF THE HIGHER PLANTS IN RELATION TO CONCENTRA- TION OF OXYGEN AND CARBON DIOXIDE.

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INTRODUCTION.

It has long been known that ethyl alcohol appears as a product of metabolism when many higher plants (including apples) are kept in atmospheres without oxygen [Lechartier and Bellamy, 1872; Pasteur, 1872; Pfeffer, 1900]. More recently an enzyme system essentially similar to the zymase of yeast has been isolated from the tissues of higher plants (including apples) [Palladin and Kostytscheff, 1906; Stoklasa, Ernest, and Chockensky, 1907, 1 and 2], and it is now generally accepted that in the usual respiration of glucose by higher plants this zymase system co-operates with an oxidation enzyme system to produce carbon dioxide. So long as the oxidation system maintains its normal activity the zymase activity of cells will not be demonstrable, but when the oxidation system is inactivated, as through the absence of the component oxygen under anaerobic conditions, zymase activity becomes evident by the accumulation of ethyl alcohol as in the zymasic metabolism of yeast cells. Further, in the usual metabolism of yeast small quantities of acetaldehyde are always produced [Fernbach and Schoen, 1920; Harden, 1923]; and small quantities of acetaldehyde have been detected as accumulating, together with ethyl alcohol, in poplar flowers placed under anaerobic conditions [Kostytscheff, Hübbenet, and Scheloumoff, 1913] and also when a maceration of germinating pea seeds acts upon glucose solution under anaerobic conditions [Neuberg and Gottschalk, 1924]. In this paper the appearance of acetaldehyde, in addition to ethyl alcohol and carbon dioxide, amongst the end products of the zymasic metabolism of apples under anaerobic conditions is recorded. Neuberg and his co-workers by following quantitatively the production of ethyl alcohol and acetaldehyde in the metabolism of yeast under different conditions, have brought strong evidence to support the original contention of Kostytscheff that acetaldehyde is the immediate

precursor of ethyl alcohol in alcoholic fermentation [see Fernbach and Schoen, 1920]. The author has adapted the technique used by Neuberg to study quantitatively the accumulation with time of ethyl alcohol and acetaldehyde in apples under different conditions. Such studies evidently give a direct measure of the extent to which zymasic metabolism occurs in the tissues of higher plants. Plant physiologists have studied the respiratory metabolism of higher plants mainly by observing the rates of gaseous exchange and by determining the values of the $\frac{\text{Carbon dioxide}}{\text{Oxygen}}$ ratios; by these methods they have shown that carbon dioxide in certain concentrations may retard the rate of production of carbon dioxide by plant tissues [Jodin, 1897; Kidd, 1917]. By determining the acetaldehyde and ethyl alcohol accumulations in apples in different gases and gas mixtures, the interesting discovery can now be recorded that carbon dioxide—above certain concentrations—even in the presence of abundant oxygen inactivates the oxidation system in apple cells without affecting the zymase system: in such concentrations of carbon dioxide, therefore, the metabolism of apple cells is essentially of a zymasic type. For convenience, the term CO_2 -zymasis will be used to describe this zymasic metabolism induced by carbon dioxide in the presence of oxygen. Also, we will speak of the zymasic metabolism occurring in the absence of oxygen as anaerobic zymasis. CO_2 -zymasis differs from anaerobic zymasis in pure nitrogen or in pure CO_2 by the greater accumulation of acetaldehyde, and the correspondingly smaller accumulation of ethyl alcohol in the former, while the total destruction of sugar appears to be the same initially.

Acetaldehyde is more toxic than ethyl alcohol; to its greater accumulation in atmospheres containing carbon dioxide (above a certain concentration) plus oxygen may probably be attributed the greater toxicity of such gas mixtures compared with pure nitrogen, pure carbon dioxide, or any mixture of nitrogen and carbon dioxide [Kidd and West, 1923]. Certain very severe cases of damage to large quantities of apples, during overseas transport under cold storage, have recently been traced to the toxicity of mixtures of carbon dioxide and oxygen, and it was, in fact, to elucidate this special toxicity and also to determine other causes of premature death of apples which have been attributed to the accumulation of toxic metabolites, that this investigation was commenced¹.

SECTION 1. THE IDENTIFICATION OF ACETALDEHYDE AND ETHYL ALCOHOL AS PRODUCTS OF THE RESPIRATION OF APPLES IN CERTAIN GASEOUS ENVIRONMENTS.

A. (i) *Demonstration of aldehyde production with intact apples.* Aqueous acid solutions of phloroglucinol (1 % phloroglucinol in 6 % sulphuric acid was

¹ This work was taken over from the Hon. Mrs Wheldale Onslow, to whom the idea of investigating the acetaldehyde and ethyl alcohol accumulation under anaerobic conditions is due, and I am greatly indebted to her for the close touch she has maintained with the research during its course.

used in these experiments) speedily combine with aliphatic aldehydes to produce sparingly soluble substances known as phloroglucides, and with excess of phloroglucinol the amount of precipitation is proportional to the amount of aldehyde present (see Appendix). These facts were exploited for the investigation of the apples. Gas mixtures of different composition were passed at the same rates for the same time-period through desiccators, containing equal numbers of apples (Fen Newtowns), placed in a room maintained at 15°. The emerging gas passed through the acid phloroglucinol reagent. The relative production of volatile aldehydes by apples under the different gaseous conditions was gauged by comparing the bulks of the phloroglucide precipitates obtained. With this technique the aldehyde production cannot be of secondary origin due to injury or to high temperatures.

Composition of gas stream	Result
Air (freed from CO ₂)	Very slight precipitation
Oxygen (freed from CO ₂)	
Nitrogen (freed from CO ₂ but contains a trace of oxygen)	Copious "precipitation"
Carbon dioxide 50 % + oxygen 50 %	" "

! The identity of these aldehydic metabolic products had to be established by other methods, as phloroglucides are amorphous substances of ill-defined melting points and of uncertain composition. Using the British apple named above, the author repeated and confirmed the experiment of Power and Chesnut [1920], who used the delicate Rimini reaction to show that acetaldehyde emanates from ripe American apples taken from an air store. So the slight precipitation of phloroglucide in the air and oxygen conditions was at least in part due to the presence of acetaldehyde in the living apples.

(ii) *Evidence that acetaldehyde is the only aldehyde produced in Exp. A (i).* The identity of the volatile aldehyde (or aldehydes), accumulating in living apples under the conditions described above, was determined by re-distilling the steam distillate of the tissue, and preparing from the first fraction crystalline derivatives with *p*-nitrophenylhydrazine [for details, see Dakin, 1908] and with dimethylhydroresorcinol [see Neuberg and Reinfurth, 1920]. *p*-Nitrophenylhydrazones. After one recrystallisation from dilute alcohol, crystals were obtained of melting point 128·5° identical with that of acetaldehyde *p*-nitrophenylhydrazone synthesised in the laboratory. The m.p. was not altered by mixing the two derivatives.

Dimethylhydroresorcides. After one recrystallisation from dilute alcohol, crystals were obtained of melting point 139–140° identical with that of acetaldehyde dimethylhydroresorcide synthesised in the laboratory. The m.p. was not altered by mixing the two derivatives. As these derivatives were so easily purified, and as no other aldehyde was identified in the first or later fractions, it was concluded that acetaldehyde alone caused the phloroglucide precipitations described in A (i).

B. *Demonstration of simultaneous ethyl alcohol production.* The steam distillates obtained as described in A (ii) were freed from acetaldehyde—by

combining this substance with *m*-phenylenediamine hydrochloride (see Appendix)—and redistilled, yielding liquids giving no reactions for ketones, but giving the iodoform reaction.

C. *Conclusions.* Air-stored apples contain traces of acetaldehyde and ethyl alcohol. These substances may accumulate in apples when there is a shortage of oxygen, or under the influence of carbon dioxide in the presence of sufficient oxygen. In the following sections the results of quantitative studies on these facts will be given and discussed.

SECTION 2. THE NORMAL ACETALDEHYDE AND ETHYL ALCOHOL CONTENTS OF SOUND APPLES STORED IN AIR.

The figures in the following table represent grams acetaldehyde found in 100 g. fresh weight of tissue of apples stored in air for different periods from October at 1°, 15° and 22°.

Table I. (See Figs. 1, 2, 3, and 4, air value lines.)

Acetaldehyde in apples kept in air.

Month	1°	15°	22°
October (experiment begun)	0.0003	0.0003	0.0003
December	0.0007	0.0005*	0.0003*
February	0.0005*	0.0007	0.0005
July	0.0006	—	—

The details of the analytical procedure will be found in the appendix.

* Colour change from green to yellow was completed. After February all apples at 15° and 22° were damaged by fungi.

Air-stored apples always contain a little acetaldehyde (see Section 1) which does not increase in amount with extension of the storage period: this confirms the results of Müller-Thurgau and Osterwalder [1915]. The average of the results given above (0.0005 g.) is used in the following sections as the air-value of acetaldehyde in apples.

Power and Chesnut [1920] always detected ethyl alcohol in steam distillates from air-stored apples. In the present experiments, estimations made in June and August, on apples stored at 1° from the previous October, gave at both times 0.006 g. ethyl alcohol in 100 g. fresh weight of apple tissue. As acetaldehyde formation has always been found to accompany the formation of ethyl alcohol in apples, and as acetaldehyde does not accumulate during storage, it is concluded that ethyl alcohol does not accumulate, and that 0.006 % may be taken as its average air value in apples. Probably both air values are too high, as aldehydic and alcoholic substances may form to some extent from apple pulp decompositions at high temperatures, and certainly the volatile esters and terpenes always present in apples will, in part, be oxidised to volatile acids in the estimation of ethyl alcohol as acetic acid (see Appendix for technique).

SECTION 3. THE PRODUCTION OF ETHYL ALCOHOL AND ACETALDEHYDE BY APPLES UNDER ANAEROBIC CONDITIONS IN AN ATMOSPHERE OF NITROGEN.

Apples were placed in closed desiccators, containing potash, which were first evacuated and then re-filled with nitrogen from a gas cylinder. Gas analysis with a Haldane apparatus showed that no carbon dioxide accumulated and no oxygen leaked in. The desiccators were placed in rooms at the desired experimental temperature (1°, 15°, or 22°), and the ethyl alcohol and acetaldehyde content of the apples at the end of definite time periods was determined (see Appendix). The results are expressed in Table II as g. substance in 100 g. fresh weight of apple tissue.

Table II. (See Figs. 1 and 2.)

Ethyl alcohol and acetaldehyde in apples kept in nitrogen.

Number of days of anaerobiosis	22°		15°		1°	
	Ethyl alcohol	Acet- aldehyde	Ethyl alcohol	Acet- aldehyde	Ethyl alcohol	Acet- aldehyde
0	0.0060	0.0005	0.0060	0.0005	0.0060	0.0005
1	—	0.0030	—	0.0013	—	—
2	0.217	0.0052	—	0.0023	—	—
3	—	0.0051	—	0.0027	—	—
4	—	0.0063 (<i>d</i>)	—	0.0030	—	—
5	0.368 (<i>d</i>)	—	—	0.0031	—	—
6	—	—	—	0.0041	—	0.0013
7	—	0.0055	—	—	0.096	—
9	—	—	0.396	—	—	—
10	—	—	—	0.0039 (<i>d</i>)	—	0.0028
11	0.664 (<i>D</i>)	0.0057 (<i>D</i>)	—	—	—	—
13	—	—	0.504 (<i>d</i>)	0.0037	—	0.0034
14	—	0.0055	—	—	—	—
15	—	—	—	—	0.170	—
17	—	—	—	—	—	—
19	0.699	—	—	—	—	0.0038
21	—	—	0.829	0.0041	—	—
27	—	—	—	—	0.278	—
30	—	—	—	0.0044	—	0.0037
35	0.663	—	—	—	—	—
38	—	—	—	—	0.389 (<i>d</i>)	—

(*d*) Protoplasmic disorganisation of superficial cells commences and continues progressively inwards, until:—

(*D*) Protoplasm of all cells of apple tissue is disorganised. Apples are completely dead.

(i) *The acetaldehyde—ethyl alcohol equilibria in anaerobic zymasis at different temperatures.* The data of Table II show that the anaerobic zymasis of apples resembles the usual metabolism of the yeast cell in that small amounts of acetaldehyde and relatively large amounts of ethyl alcohol are afterwards found present. Harden [1923] writes of yeast: "This of course coincides with the occurrence of acetaldehyde as an intermediate product of alcoholic fermentation." The view is adopted here, that in the anaerobic zymasis of apple cells acetaldehyde is the immediate precursor of ethyl alcohol to which it is reduced by the activity of a reductase system. Its accumulation in these experiments, and in the experiments mentioned in the Introduction to this

paper on poplar flowers and peas, cannot yet be precisely explained, but may in some way be associated with the attainment by the reductase system of maximal activity under anaerobic conditions. The gradual flattening of the progress curves for acetaldehyde formation (Fig. 2) would then represent the attainment of equilibria, between aldehyde and alcohol, which are reached more rapidly at higher than at lower temperatures.

Fig. 1. Production of ethyl alcohol by apples in anaerobiosis (see Table II).

Fig. 2. Production of acetaldehyde by apples in anaerobiosis (see Table II).

It seemed possible at first that the escape of acetaldehyde from apple tissue might account for the flattening of the curves, and to investigate this possibility the phloroglucinol technique (see Appendix) was used to show that the escape of acetaldehyde from aldehyde-laden apples, submitted at 1° for one week to a gas stream from a nitrogen cylinder, is not significant at this temperature. The escape in a closed space will be very much less, and since at 1° the flattening of the aldehyde curve is not an index of the evaporation of acetaldehyde, at all temperatures, some other explanation had to be found. Nor is the upper limit due to toxic arrest, for no universal correlation

is observed between the apparent cessation of acetaldehyde accumulation and the disorganisation of the protoplasm, since accumulation at 1° ceased after 15 days, but no injury to cells was visible even after 30 days. The possibility that acetaldehyde is formed from some substance, other than glucose, present in apples only in small quantities gets no support, as different amounts of acetaldehyde are formed at different temperatures.

(ii) *The rates of production of acetaldehyde and ethyl alcohol in anaerobic zymasis.* The initial rates of production are some direct function of the temperature; for example, during the first 48 hours the approximate average rates of production of ethyl alcohol in mg. per hour are, at 22° 4.4, at 15° 1.8, and at 1° 0.6. Retardation of these initial rates and final cessation of acetaldehyde accumulation and of ethyl alcohol production occur more rapidly at the higher temperatures. This retardation in respect of acetaldehyde has just been discussed, and attention will now be focussed on the alcohol progress curves. Retardation and ultimate cessation of alcohol production in nitrogen is correlated with the protoplasmic disorganisation which commences in the superficial cells, and which gradually becomes more profound until all the cells of all the apples under experimental conditions are dead. In this connection it is interesting to note that Lechartier and Bellamy [1872] discovered that sooner or later many fruits—including apples—ceased to produce carbon dioxide under anaerobic conditions. This cessation probably synchronises with the cessation of ethyl alcohol production on the death of the cells. That anaerobic zymasis occurs only in living cells was further shown by the observation that no increase occurred in the acetaldehyde or ethyl alcohol content of apples previously killed by prolonged freezing (one month at -11°) and subsequently placed at 15° for seven days under anaerobic conditions. After disorganisation has commenced in apples and in other oxidase tissues under anaerobic conditions, browning will occur as a post mortem phenomenon on readmission of oxygen. The death of apple cells under anaerobic conditions is attributed to the ethyl alcohol formed, although the absence of oxygen, and the presence of the more toxic acetaldehyde probably aggravate the effect of the alcohol.

No relation is apparent between aldehyde concentration and rate of alcohol production in anaerobic zymasis; for at 22° the rate becomes markedly retarded after about three days, when the aldehyde concentration is 0.005 %, and by the eleventh day the rate has fallen to one-quarter of the initial value, whereas the aldehyde concentration has only increased to 0.006 %, and it will be shown in Section 5 that concentrations of acetaldehyde less than 0.015 % do not retard the rate of production of ethyl alcohol to any marked extent. Alcohol production in anaerobic zymasis is believed to be a toxic process; the curves for 1° and for 15° were also, without doubt, each tending towards toxic limits which possibly would have been at a higher level at 15° than at 22°, thus explaining the cutting of the curve for 22° by that for 15°.

The dotted part of the curve for 15° is not based on experimental results, but represents approximately what would have been obtained in a more prolonged experiment. At 1° the toxic concentration of ethyl alcohol must be very slowly attained, since no injury was observed until the 38th day, therefore it is not surprising that the steady rate of production of ethyl alcohol did not alter during the period of the experiment.

SECTION 4. THE PRODUCTION OF ETHYL ALCOHOL AND ACETALDEHYDE BY APPLES UNDER THE INFLUENCE OF CARBON DIOXIDE, IN THE PRESENCE OF OXYGEN: CO₂-ZYMESIS.

Carbon dioxide and oxygen, after being mixed in the desired proportions in gasometers of 60 cubic feet capacity, were passed over apples, placed at 15° in desiccators, at a rate sufficient to prevent any appreciable accumulation of carbon dioxide around the apples from their respiration. It has been shown in Section 1 that zymasic metabolism may occur under these conditions, and this has been followed quantitatively by determining the acetaldehyde accumulation after different time-periods in each gas mixture. As the gas around the apples was moving in these experiments, in addition to estimating the aldehyde content of the apples at the conclusion of each time-period by the steam distillation technique, it was necessary to estimate the acetaldehyde escaping during each period. For this purpose the emerging gas was passed through acid phloroglucinol solution, and the weight of aldehyde in the gas current, calculated from the weight of phloroglucide obtained (see Appendix), was added to the residual aldehyde content of the apples as determined by the steam distillation technique. The results are expressed in Table III as g. acetaldehyde in 100 g. fresh weight of apple tissue. The oxygen concentration is in every experiment the percentage complement of the CO₂ concentration.

(i) *CO₂-zymesis*. It will be seen from the table that acetaldehyde is increasing with time in all these cases, whilst in air storage there is no such increase. It will be shown later, in Section 5, that alcohol is also increasing so that we have the metabolism associated hitherto with anaerobic conditions also brought about by sufficient CO₂ in spite of the presence of oxygen. This special effect, which we may call CO₂-zymesis, is probably due to CO₂ inactivating the oxidation system concerned in normal respiration without influencing zymase splitting. Willstätter and Stoll [1918] have shown that the activity of peroxidase systems is diminished to one-half in water saturated with carbon dioxide, though no destruction of enzyme occurs, and their observations are of interest in showing that carbon dioxide has an inhibiting effect on at least one oxidation system. As the apple tissue used never gave p_H values > 4, no connection can exist between the phenomena recorded and the concentration of hydrogen ions, since carbon dioxide will not alter a p_H so much on the acid side of neutrality. Without attempting to analyse the

Table III. (See Fig. 3.)

Acetaldehyde formation in apples by CO₂-zymasis at 15°, in relation to concentration of carbon dioxide.

Days in gas mixture	Experimental Series 1 (Spring)						Experimental Series 2 (Summer)		
	12 % CO ₂	20 % CO ₂	30 % CO ₂	50 % CO ₂	70 % CO ₂	100 % CO ₂	50 % CO ₂	95 % CO ₂	99 % CO ₂
	I	II	III	IV	V	VI	VII	VIII	IX
0	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
1	—	—	—	0.003	0.003	0.003	—	—	—
2	—	—	—	—	0.005	0.004	—	—	—
3	—	—	0.001	0.004	0.004	0.005	0.015	—	—
4	—	—	—	0.009(A)	—	—	—	—	—
5	—	—	—	—	0.010(A)	—	—	0.036(d)	0.006
6	—	—	0.006	—	—	—	0.038(d)	—	—
7	—	0.003	—	—	—	—	—	—	—
8	—	0.001	—	—	0.019	—	—	0.052	0.015(d)
9	—	—	0.012(d) (A)	—	—	—	—	—	—
11	—	—	—	0.028(d)	0.030(d)	0.006	0.058(A)	—	—
12	—	—	0.033	—	—	—	—	0.057(A)	0.014
15	—	—	—	—	—	—	0.055	0.054(D)	0.025
16	—	0.015(d)	—	—	—	—	—	—	—
17	—	0.012	—	—	—	—	—	—	—
20	—	—	—	—	—	0.006(d)	—	—	—
21	0.001	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	0.057(D)	—	—
29	0.002	—	—	—	—	—	—	—	—
50	0.012(d)	—	—	—	—	—	—	—	—

(A) Browning of cells near the apple cora noticed (Brownheart [Kidd and West, 1923]).
(d) Protoplasmic disorganisation (with browning, when oxygen is present) of superficial cells commences and continues progressively inwards (Deep scald [Kidd and West, 1923]), until:—
(D) Protoplasm of all cells of the apple is completely disorganised (apple is completely brown, when oxygen is present).

Fig. 3. Acetaldehyde formation in apples by CO₂-zymasis at 15° in relation to concentration of CO₂ (see Table III).

cause of this action of carbon dioxide, we will consider certain aspects of the effects produced, and the first point is that there seems to be a lower limit of concentration of CO_2 below which CO_2 -zymasis is not produced. For these "Newtown Wonder" apples this is placed tentatively at 10 % CO_2 , as no increase was observed in the acetaldehyde content of apples which had been stored at 8° for six months in a gas mixture of 10 % CO_2 , 10 % oxygen, 80 % nitrogen, but in this connection we shall see later in this section that future research may necessitate a change in our views. The second point is that the co-operation of oxygen is needed for CO_2 -zymasis to occur, as anaerobic zymasis, of the type described in Section 3, occurs in pure CO_2 . Anaerobic experiments were set up precisely as described in the last section, but using pure CO_2 instead of pure N_2 . With mixtures of CO_2 and O_2 between these limits the effect is well marked but the precise nature of the relation is complicated by the senescent state of the apples and will be discussed later (see p. 937). The third point is that much more acetaldehyde accumulates in the tissues under CO_2 -zymasis than in anaerobic zymasis. This will be considered further in Section 5 where the alcohol production of the two states is compared, but we note here the difference between curves IV, V, VII and VIII in Fig. 3, and the anaerobic curves whether the gas environment is pure nitrogen (Fig. 2) or pure carbon dioxide (Fig. 3, curve VI). When the external oxygen concentration is between zero and 5 %, observed effects are intermediate between CO_2 -zymasis and anaerobic zymasis (Fig. 3, curve IX), and these are to be expected, as evidently it is the concentration of oxygen in the intercellular spaces that determines which type of zymasis will occur in different localities in a given apple, and these concentrations will probably, in a bulky tissue such as an apple, be smaller in the localities furthest removed from the lenticels and other points at which oxygen enters from the outside atmosphere.

(ii) *Progress curves for acetaldehyde formation in CO_2 -zymasis.* Curve VII (which cannot, on the results obtained, be distinguished from curve VIII) represents a typical progress curve for acetaldehyde formation in CO_2 -zymasis, and curve IV (which cannot, on the results obtained, be distinguished from curve V) represents the initial phase of acetaldehyde accumulation under similar CO_2 concentrations, but at an earlier stage in the storage season when the apples are less senescent. By comparing curves VII and VIII or curves IV and V we see that at a given stage of the progressive senescence during storing, provided that more than 5 % oxygen is present, the rate and extent of CO_2 -zymasis, and the incidence of the (*d*) condition of superficial browning appear to be the same in all concentrations of CO_2 greater than 50 %. By comparing curve IV with curve VII we see that for a given concentration of CO_2 (50 % in this case), the rate of CO_2 -zymasis must increase as the senescence of apples advances in the later stages of the storage season.

The oxidase browning mentioned above is caused by the disorganisation

of the protoplasm in the superficial cells in an atmosphere containing oxygen, and the flattening of the progress curves for acetaldehyde production in CO_2 -zymasis proceeds hand in hand with the increasing profundity of tissue-browning, in striking contrast with the flattening of the aldehyde curves in anaerobic zymasis (Fig. 3, curve VI and curves in Fig. 2) where cessation of acetaldehyde accumulation occurs before the disorganisation of protoplasm commences. Further, progressive injury and death of cells from the surface inwards (Deep scald [Kidd and West, 1923]) occur more rapidly in CO_2 -zymasis than in anaerobic zymasis, and we notice in CO_2 -zymasis a type of injury (*A*), described as browning of cells near the apple core, which does not occur in anaerobic zymasis (Brownheart [Kidd and West, 1923]). Although Brownheart may occur under the influence of CO_2 without acetaldehyde formation [Kidd and West, 1923], the author definitely attributes the more rapid death of superficial cells in CO_2 -zymasis to the greater quantities of the toxic acetaldehyde produced. These matters will be further discussed in the next section; here we note that whereas concentrations of 0.004–0.006 % acetaldehyde, found under anaerobic conditions, are not injurious to apples, concentrations of 0.03–0.05 % retard CO_2 -zymasis and finally cause its cessation at the death of the tissue.

(iii) *CO_2 -zymasis in relation to the concentration of CO_2 and the resistance of individual apples.* We have seen that early in the storage season the reaction of a given apple to CO_2 , as shown by the rate of aldehyde accumulation, must be different from its reaction at a later period. This difference is now attributed to the diminishing resistance to CO_2 with the increasing senescence of the apple. We will consider the behaviour of apples in concentrations of CO_2 between 10 and 50 % and it will shortly be obvious that, at a given period in the storage season, the apples used in these experiments must have been in different senescent states as judged by their varying resistances to such concentrations.

Table IV. *Comparison of the average acetaldehyde contents of non-browned apples and browned apples in samples previously identically treated.*

% CO_2	Days in gas	Number of non-browned apples	% acetaldehyde in non-browned apples	Number of browned apples	% acetaldehyde in browned apples
50–75	11	—	—	6	0.030
30	9	2	0.0004	4	0.018
30	12	—	—	6	0.033
20	16	3	0.0003	3	0.036
20	17	2	0.0002	4	0.023

It has already been shown that as a result of acetaldehyde production in CO_2 -zymasis the surfaces of apples become brown, and it was noticed, after certain time-periods in concentrations of CO_2 of 20 and 30 %, that whereas some of the apples in the experimental samples of six appeared to be brown, others still appeared to be healthy. In Table IV are recorded the results of separate analyses of such browned and non-browned classes in

samples of apples previously identically treated. It was in some cases difficult to judge whether apples were brown or non-brown; but when an apple, on cutting, gave the characteristic smell associated with zymasis it was included in the browned class, although it is very probable that in some instances zymasis had been of shorter duration than in the obviously browned apples. Separate analyses of individual apples in samples have not yet been made.

These figures prove that the apple population used in all the experiments described in this paper must be considered as mixed, since some of the apples under 30 % CO_2 for nine days had accumulated acetaldehyde whereas in at least two apples no CO_2 -zymasis had occurred, and other samples under 20 % CO_2 for 16 and 17 days gave even more striking figures. Indeed, assuming a linear relation, calculation shows that some apples will in nine days have accumulated 0.02 % acetaldehyde in 20 % CO_2 , and we have just noticed that in other apples no zymasis occurred during this period in 30 % CO_2 ; however, it will be made clear later that a given apple responding to 20 % CO_2 will respond to 30 % CO_2 even more rapidly. Winkler [1923] has shown that apples picked from different sides of the same tree subsequently remain healthy for different lengths of time in air storage at low temperature, and there are many other observations to show that mixed effects are to be expected unless special precautions, as to aspect, age of tree, etc., are taken in picking apples and no such precautions were taken in picking the "Newtown Wonder" apples used in these experiments. These mixed effects in relation to CO_2 result from the differences in resistance to CO_2 shown by individual apples which have, in equal periods of storage, reached different stages of senescence owing, at least in part, to the differences in their orchard histories. Taking the time required for CO_2 -zymasis to commence in an apple as a direct measure of its resistance to a given concentration of carbon dioxide, it seems probable from Table IV that the scatter of the resistances of individual apples in a given sample decreases as the incident CO_2 concentration increases. Thus for 20 % CO_2 the figures in Table IV indicate a wide scatter of resistances, as some apples must have commenced zymasis at an early stage whereas others show no zymasis even after 17 days. We have not sufficient data to compare the effects of 30 % CO_2 with 20 % CO_2 , but there is evidently a fairly wide scatter of resistances to 30 % CO_2 and in concentrations of 75 % CO_2 no differences in resistance have been detected by the methods of analysis employed. With more accurate observations it seems certain that a small scatter of resistances would be detected for all concentrations, the scatter always becoming less as the CO_2 concentration is increased.

From consideration of the average accumulation of acetaldehyde in CO_2 -zymasis in given samples in different concentrations of CO_2 as shown in Fig. 4, it is believed that in all concentrations of CO_2 the average initial rate of zymasis increases at first slowly and then more rapidly until the toxic effect of the acetaldehyde comes into play in all cases, causing retardation and finally cessation of production as shown in curves VII and VIII. Thus

progress curves I, II and III would have been definitely sigmoid in shape if observations had been made after longer times with 12 % CO₂, 20 % CO₂ and 30 % CO₂. No figures for the early periods in concentrations of CO₂ > 50 % are available; progress curves have, however, in conformity with the ideas just expressed, been drawn in slightly sigmoid form; but no attempt has been made to indicate the beliefs that with more accurate measurements, firstly, curve V for 70 % CO₂ in the spring would have been slightly nearer the *y* axis than curve IV for 50 %, and curve VIII for 95 % CO₂ in the summer would have been slightly nearer the *y* axis than curve VII, and secondly, with the higher concentrations of CO₂ the more nearly will progress curves approximate to a logarithmic form. Comparing curves I, II, III and IV, we see that as the concentration of CO₂ increases from 12 % to 50 % the forms of progress curves change from sigmoid with longer bases to sigmoid with shorter bases, thus gradually approaching a logarithmic form¹.

Evidence will now be presented that at a given time in the storage season, once the resistance of an individual apple has been overcome, CO₂-zymasis will occur in an apple at the same rate and to the same extent independently of the inducing concentration of CO₂. Firstly, there is the obvious evidence of curves IV and V and curves VII and VIII which, for we now see that they are not in theory coincident, must be close and parallel to one another; evidently the resistances of all apples are very speedily overcome under concentrations of CO₂ > 50 %, so that raising the concentration of CO₂ from 50 to 95 % does not appreciably alter the average rate or the average extent of zymasis in a sample. Secondly, it will be noticed in Table IV that in one experiment with 30 % CO₂, by chance, six apples of low resistance were used and a similar amount of acetaldehyde had accumulated in them during 12 days as would have been expected under concentrations of CO₂ greater than 50 %.

At least two causes may be important in overcoming the resistance of an individual apple to a given concentration of CO₂; firstly, there is the cumulative effect of CO₂ with time, and secondly, there is the increasing senescence of the apples. Considering the second factor, it is evident that, under the experimental conditions employed, the advance of senescence of the apples—which had previously been kept for six months at 1°—must have been accelerated by removal to 15°, and also by the high concentrations of oxygen used in conjunction with the lower concentrations of CO₂. With 12 % CO₂ (curve I), the oxygen concentration surrounding the apples was 88 %, and only slight zymasis had occurred after 30 days, although we see from Table III that during 50 days considerable zymasis had occurred. Evidently there are many

¹ These effects fall into the same schema as the experiments of Henderson Smith [1921] on the killing of *Botrytis* spores by phenol. In these experiments, however, no attempt has been made to investigate the effect of varying concentrations of CO₂ on the rate of killing of apples; attention has been fixed on the toxic chemical changes intermediate between the commencement of the reaction to CO₂ and the death of the apples.

points which require further elucidation, for although it has already been reported in paragraph 1 of this section that apples have been kept for six months in a gas mixture of 10 % CO_2 , 10 % oxygen, 80 % nitrogen at 8° without showing zymasis (Fig. 3, line X), it seems possible that, with greater oxygen concentrations and also at a higher temperature (15°), the senescent states of apples may so advance that 10 % CO_2 or less might, given time, overcome some of the individual resistances of apples and thus induce zymasis.

SECTION 5. A QUANTITATIVE COMPARISON OF ANAEROBIC ZYMASIS AND CO_2 -ZYMASIS.

. For anaerobic zymasis we have the data of Table II from experiments carried out in closed desiccators. For comparable experiments on CO_2 -zymasis, apples were placed at 1° in closed desiccators containing gas of the initial composition of 50 % CO_2 + 50 % O_2 , and after definite time periods their acetaldehyde and ethyl alcohol contents were determined by the steam distillation technique (see Appendix). After the longest period (34 days) the gas composition in the desiccators was 78 % CO_2 + 22 % O_2 . It has been shown in Section 4 that such an increase in CO_2 concentration does not appreciably influence acetaldehyde production, and therefore probably has no effect on alcohol production. In Table V the analyses of these apples are

Table V. (See Fig. 4.)

Comparison of ethyl alcohol and acetaldehyde production in apples by CO_2 -zymasis and anaerobic zymasis.

Days in gas	Gas mixture initially 50 % CO_2 + 50 % O_2 rising to 78 % CO_2 + 22 % O_2		Nitrogen gas	
	Ethyl alcohol	Acetaldehyde	Ethyl alcohol	Acetaldehyde
0	0.0060	0.0005	0.0060	0.0005
3	—	0.010	—	—
5	0.046	—	—	—
6	—	—	—	0.0013
7	—	0.021 (A)	0.096	—
8	0.056 (A)	—	—	—
10	—	—	—	—
13	—	—	—	0.0028
15	—	0.032 (d)	0.170	0.0034
16	0.079 (d)	0.037	—	—
19	—	—	—	0.0038
23	0.084	—	—	—
25	—	0.042 (D)	—	—
27	—	0.040	0.278	—
28	—	—	—	0.0037
32	—	0.040	—	—
34	0.080 (D)	—	—	—
38	—	—	0.389 (D)	—

(A) Browning of cells near the apple core noticed.

(d) Protoplasmic disorganisation (with browning, when oxygen is present) of superficial cells commences and continues progressively inwards, until:—

(D) Protoplasm of all cells of the apple is completely disorganised (apple is completely brown, when oxygen is present).

contrasted with data for anaerobic zymasis at 1°. Since acetaldehyde accumulation is of the same order in pure CO₂ (Table III, column V) as in pure nitrogen (Table II), Table V may be taken to illustrate the influence of oxygen on the nature and amounts of the products of CO₂-zymasis. The results are expressed as g. acetaldehyde in 100 g. fresh weight of apple tissue.

(1) *Comparison of the $\frac{\text{alcohol}}{\text{aldehyde}}$ ratios after anaerobic zymasis and CO₂-zymasis.*

It will be seen that though concentrated CO₂ can produce zymasis in the presence of oxygen yet the resulting accumulation of aldehyde and alcohol does not follow the same course as with zymasis in anaerobic conditions. The proportions of alcohol and aldehyde differ greatly in the two cases. A comparison of curves I with curves II in Fig. 4 shows that in CO₂-zymasis, in correlation with the presence of oxygen, considerably more acetaldehyde and correspondingly less ethyl alcohol accumulate than in anaerobic zymasis, and this point is emphasised in the following table in which the ratios of ethyl alcohol to acetaldehyde accumulations at different times in the experiments are given.

Days	Anaerobic zymasis $\frac{\text{alcohol}}{\text{aldehyde}}$	CO ₂ -zymasis $\frac{\text{alcohol}}{\text{aldehyde}}$
5	65	2.6
10	50	2.0
20	54	2.4

This increased accumulation of acetaldehyde in CO₂-zymasis cannot be attributed to the oxidation of ethyl alcohol by oxygen, as it will be shown in Section 6 that apple tissue does not effect such an oxidation. The author believes that the difference between the two types of zymasis is due to the effect of oxygen on the reductase system controlling the reduction of acetaldehyde to ethyl alcohol, the final step in the zymase cleavage of glucose. Under anaerobic conditions as soon as the reductase system attains maximal activity acetaldehyde accumulation ceases. In CO₂-zymasis the continuous presence of more than 5% oxygen retards the reduction of acetaldehyde to ethyl alcohol, so acetaldehyde accumulates as long as the zymase system remains active, and the amount of ethyl alcohol formed is correspondingly reduced.

(2) *A comparison of the rates of anaerobic zymasis and CO₂-zymasis.* The initial rates of anaerobic and CO₂-zymasis were apparently identical since in the former the percentage accumulation of alcohol + aldehyde was 0.054% after five days, and in the latter 0.052%. During the period of the experiment the rate of anaerobic zymasis (as measured by alcohol production) was not retarded to any marked extent, nor was any injury observed until 40 days had passed. It was quite otherwise in CO₂-zymasis, as after five days the rates of formation of both ethyl alcohol and acetaldehyde were retarded progressively with the disorganisation of protoplasm as described in Section 3, paragraph (ii). CO₂-zymasis occurs only in living cells, for, apart from the obvious evidence of the

curves II in Fig. 4, the author, by prolonged freezing, killed apples, then placed them in 50 % CO_2 + 50 % oxygen for seven days and noticed that neither alcohol nor aldehyde was produced. This accelerated death of apple cells in atmospheres of carbon dioxide + oxygen has already, in the last section, been attributed to the increased acetaldehyde accumulation under these conditions.

In conclusion, the influence of acetaldehyde on the rate of production of ethyl alcohol in CO_2 -zymasis will be considered. Assuming that low concentrations of acetaldehyde have no retarding effect, we may as a standard for comparison represent the initial constant rate by the figure 100. That in the later stages of zymasis an inverse ratio seems to exist between the aldehyde concentration in the apples, and the rates at which alcohol is produced, is indicated in the following table where these rates, calculated in terms of the standard from the slopes of the tangents drawn at different points on the alcohol progress curve II in Fig. 4, are shown as decreasing regularly to zero as the acetaldehyde concentration increases.

Fig. 4. Comparison of ethyl alcohol and acetaldehyde production at 1° in CO_2 -zymasis and in anaerobic zymasis (see Table V).

Acetaldehyde concentration in g. in 100 g. fresh weight of tissue   Relative velocity of alcohol formation in CO₂-zymasis

0.0005 – some value <0.017	100 (standard)
0.017	65
0.023 (<i>A</i>)	52
0.028	43
0.034 (<i>d</i>)	24
0.038	13
0.043 (<i>D</i>)	3.0

(*A*), (*d*) and (*D*) as in Table V.

SECTION 6. QUANTITATIVE EXPERIMENTS PROVING THAT APPLE TISSUE DOES NOT OXIDISE ETHYL ALCOHOL OR ACETALDEHYDE.

If, as is generally accepted, the usual oxidation of glucose by higher plants is effected by the co-operation of a zymase and an oxidation system, evidently ethyl alcohol or at least one of its precursors must be substances oxidisable by plant cells. Space does not permit of an adequate discussion here of theories of the biochemistry of sugar oxidation, but mention must be made of Kostytscheff's observation [1909] that ethyl alcohol is not oxidised to carbon dioxide by higher plants, and of the experiments of Ciamician and Ravenna [1918] who showed that crushed spinach leaves, which can effect many interesting oxidations, will not oxidise either ethyl alcohol or acetaldehyde. The following experiments provide further evidence that tissues of higher plants cannot oxidise either ethyl alcohol or acetaldehyde and therefore some precursor of acetaldehyde must be oxidised to produce carbon dioxide in aerobiosis. Apples were kept under anaerobic conditions at 15° for definite periods, when the alcohol and aldehyde accumulations were determined by analysing samples. The remaining apples were analysed after further periods in CO₂-free air, or CO₂-free oxygen.

Days in nitrogen	Subsequent days in air	% ethyl alcohol
9	0	0.396
9	7	0.450
9	14	0.420

We notice that there is no loss of ethyl alcohol either by oxidation or evaporation. Similar experiments were carried out for acetaldehyde: the tabulated results show that there is no loss of acetaldehyde either by oxidation or evaporation. Further as there is no increase in acetaldehyde, ethyl alcohol is not oxidised to acetaldehyde by apple tissue in the presence of oxygen (Section 5, paragraph (i)).

Days in nitrogen	Subsequent days in air	Subsequent days in oxygen	% acetaldehyde
3	0	0	0.0027
3	8	0	0.0025
3	0	6	0.0028
3	0	13	0.0025

SUMMARY.

1. Neither ethyl alcohol nor acetaldehyde accumulates as intermediate or end product of the respiration of apple cells in air; nor can either of them be oxidised by apple tissue.

2. In the absence of oxygen, respiration is of a zymasic type, ethyl alcohol and traces of acetaldehyde accumulating in apple cells. The term anaerobic zymasis is used to describe the effect.

3. Carbon dioxide even in the presence of abundant oxygen may cause the respiration of apple cells to be changed to a zymasic type. The term CO_2 -zymasis is used to describe the effect. Whereas CO_2 -zymasis commenced nearly immediately in all the apples used in these experiments under concentrations of $\text{CO}_2 > 50\%$, a wide scatter of resistances to concentrations of CO_2 between 10 and 30 % was noticed.

4. In CO_2 -zymasis, associated with the presence of oxygen, the ratio ethyl alcohol/acetaldehyde is 2/1, contrasting strikingly with the ratio 50/1 obtained in anaerobic zymasis.

5. Gas mixtures of carbon dioxide and oxygen may be even more injurious to apples than anaerobic conditions, owing to the toxicity of acetaldehyde being greater than that of ethyl alcohol. The relations of these substances to the so-called physiological diseases of apples will be discussed elsewhere, in a later paper.

The analytical part of this work was carried out in the biochemical laboratory at Cambridge and I am grateful to Sir F. G. Hopkins for the facilities given, and for the interest with which he has followed the research. I express my gratitude to Dr F. F. Blackman for the general supervision he has given during the course of this research, and I also record my thanks to him and to Dr Franklin Kidd for their valuable criticisms on the interpretation and presentation of the results. The physiological part of this research was performed at the Low Temperature Research Station, Cambridge, and I thank Dr Franklin Kidd and Dr Cyril West for placing their experience, on request, at my disposal. To Mr S. G. Crouch, of this Station, I am indebted for assistance in controlling experimental conditions. I thank the Food Investigation Board for a personal grant, and for defraying all expenses incurred during the research.

APPENDIX.

MATERIAL AND METHODS OF QUANTITATIVE ANALYSIS.

Material. "Newtown Wonder" apples, grown in the locality of Canterbury on a gravel soil, were used in all the quantitative experiments which have been described. The apples were picked regardless of their aspect, so it is not surprising that in some respects they behaved as a mixed population (Section 4, paragraph (iii)). On arrival at the Low Temperature Research Station, Cambridge—two days after picking—the apples were wrapped in

papers impregnated with mineral oil and placed, in air, in a room kept at a constant temperature of 1° , and were unwrapped and removed just before each experiment started. With the apparatus available, six apples formed the most convenient sample, and this number was used in determining every figure given in the tables.

Methods of quantitative analysis.

(1) *Estimation of the acetaldehyde and the ethyl alcohol content of apples.* A steam distillation technique was used to expel the acetaldehyde and the ethyl alcohol from apples immediately after their removal from the physiological conditions of the experiment in progress. 1500 cc. of water were placed in a two litre flask, through the stopper of which passed the tapered base of a glass cylinder (of dimensions $48'' \times 2\frac{3}{4}''$) wrapped with cotton wool jackets. The water was boiled vigorously until the walls of the cylinder were very hot, and then the previously weighed apples were each cut into eight pieces and dropped into the cylinder, where they rested on a support of perforated zinc, kept in position by a rubber collar. The cylinder was then immediately connected, by means of a splash trap passing through a stopper of $2\frac{3}{4}''$ diameter, to a Liebig condenser previously connected by means of an adapter with an ice-cooled spiral condenser, the base of which passed through the stopper of a filtering flask of 1 litre capacity to a level below that of 50 cc. of liquid placed in the flask. The aqueous distillate collected in the filtering flask. To prevent loss of aldehyde or alcohol, the outlet of the filtering flask was connected with a second filtering flask by means of a bent glass tube which reached a level, in this second flask, below that of 50 cc. of liquid placed in the flask.

In estimating the acetaldehyde content of apples, 50 cc. of sodium hydrogen sulphite—of strength at least double that necessary for combination with the anticipated amount of acetaldehyde—were placed in each of the receiving flasks, and in a control flask. All the flasks were cooled by ice. Experience showed, first, that acetaldehyde only rarely escaped to the second flask, and then only in traces, and second, that after two hours' steam distillation all significant acetaldehyde was expelled from apple pulp. The amount of acetaldehyde which distilled was estimated by titrating the sodium hydrogen sulphite in the receiving and control flasks against standard iodine, using starch solution as indicator. The method, in all its essentials, is that of Ripper [1900].

In estimating the ethyl alcohol content of apples, 50 cc. of water were placed in each of the ice-cooled receiving flasks to prevent any alcohol from escaping. Three hours' steam distillation was necessary to expel all significant ethyl alcohol from apple pulp. The aqueous distillate was made up to a volume of 1 litre, and the alcohol content of each 500 cc. part was simultaneously determined by following the technique described by Neuberg and Hirsch [1919]; each part was first freed from acetaldehyde by adding

m-phenylenediamine hydrochloride, allowing to stand for half-an-hour, and then gently boiling under a reflux condenser for one hour. After cooling, the resulting brown green fluorescent solution, plus the washings from the reflux condenser, was made just alkaline with caustic soda using solid phenolphthalein as an indicator, and was subsequently distilled nearly to dryness *in vacuo*. The alcoholic distillate was collected in a filtering flask to the outlet of which was connected a bent glass tube dipping under 50 cc. of concentrated sulphuric acid placed in a second filtering flask, which was also connected by pressure tubing with a vacuum water pump. Both flasks were cooled with ice. The concentrated sulphuric acid absorbed any alcohol escaping from the main distillate by forming, for the most part, ethyl hydrogen sulphate [see Dox and Lamb, 1916]. The distillate in the first flask and 20 g. of powdered potassium dichromate were then placed in a Claisen flask connected with a Liebig condenser, and to them were slowly added through a dropping funnel the acid contents of the second flask. Complete oxidation of ethyl alcohol to acetic acid occurred in half an hour at room temperature under these conditions, and by subsequent distillation—with frequent additions of water to the Claisen flask—all the acetic acid was collected. Titration against standard caustic soda gave values from which the alcohol content of the original steam distillate was calculated.

(2) *The estimation of the acetaldehyde escape from apples.* In the experiments in which gas currents were used, the escape of acetaldehyde was gauged by the phloroglucinol method referred to in Section 1 of this paper. Counciler [1896] described the phloroglucides, formed by the condensation of the lower aliphatic aldehydes with phloroglucinol in acid solution, as amorphous compounds only sparingly soluble in the medium in which they are formed. Gravimetric estimations of formaldehyde [Clowes, 1899] and furfuraldehyde [Kröber, 1901] are based on the insolubility of their phloroglucides. Clowes does not discuss amounts of formaldehyde less than 0.05 g., and Kröber specifically limits the reliability of his method to amounts between 0.03 and 0.3 g. The author endeavoured, by applying Counciler's discovery, to evolve a method for estimating acetaldehyde, in amounts less than 0.03 g., gravimetrically as acetaldehyde phloroglucide. 10 cc. of an acetaldehyde solution (simultaneously standardised by the Ripper method) were added to 40 cc. of a solution of 1 % phloroglucinol dissolved in 6 % (by volume) aqueous sulphuric acid (it was essential in the experiments on apples to use a non-volatile acid). The phloroglucide quickly appears as a white precipitate, which on standing turns yellow. After 24 hours the phloroglucide was collected by filtration through a weighed Gooch crucible, washed with 100 cc. distilled water, and then dried for four hours in a steam-bath in which the oxygen tension was reduced by evacuation with a water-pump, as phloroglucides absorb oxygen rapidly at high temperatures. The crucible was then placed in a weighed covered vessel, cooled in a desiccator and weighed. The

following results on plotting lie approximately on a straight line, which may be used for determining acetaldehyde in quantities greater than 0.015 g.

g. acetaldehyde	g. phloroglucide
0.016	0.017
0.019	0.054
0.033	0.158
0.042	0.247

Although the method for several reasons (the discussion of which is postponed) is not recommended for work requiring a high degree of accuracy it has proved most useful in the experiments of comparison described in this paper. The conditions employed in obtaining the figures given above were, as far as possible, repeated when estimating the acetaldehyde escape from apples.

Each gas stream on emerging from the desiccators containing apples passed through 40 cc. of a solution of 10 % phloroglucinol in 6 % (by volume) sulphuric acid, to which were added 10 cc. of distilled water to make up the volume of the precipitating medium to 50 cc. After the apples had been removed from the desiccators for analysis by the steam distillation technique, the phloroglucide precipitates were treated precisely as described above, and from their weights the acetaldehyde escape (if greater than 0.015 g.) was obtained by referring to the line plotted from the results given above.

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