CCXXI. STUDIES IN ZYMASIS.
V. SEASONAL FLUCTUATIONS IN ZYMASIS AND IN
CARBON DIOXIDE/ALCOHOL NUMBER RATIOS
IN APPLES IN THE ABSENCE OF OXYGEN.

BY JOHN CARTER FIDLER.

From the Department of Botany, Armstrong College, Newcastle-upon-Tyne.

(Received July 26th, 1933.)

TOWARDS the end of the nineteenth century it was realised that the cells of many higher plants could form ethyl alcohol in the absence of oxygen [see literature quoted by Kostytschew, 1927], and several workers made simultaneous measurements of the production of ethyl alcohol and CO_2 in anaerobiosis.

Recent comparisons of anaerobic and aerobic metabolism have led to an alteration of biological outlook on cell respiration in animals, yeast and bacteria. It was shown by Blackman [1928] that the cells of higher plants fall into line with those of other organisms. Blackman based his conclusions on measurements of the CO_2 output of stored Bramley's Seedling apples in air and in nitrogen. At that time he made the primary assumption in his paper that the ratio CO_2 /ethyl alcohol in the anaerobic respiration of this variety is unity. For example, he wrote, "in nitrogen, Group D (by which he implies intermediate compounds between hexoses and ethyl alcohol) proceed quantitatively to the two final products CO_2 and alcohol in the usual ratio," and, "it will be held that for one atom of carbon thus detected (as CO_2) there are two atoms of carbon excreted into the tissues as alcohol." Actually, as will be pointed out later, the precise value of the ratio CO_2 /ethyl alcohol, although affecting his arithmetical deductions slightly, does not influence his main argument. Some of his conclusions, however, are only valid provided the ratio, whatever may be its magnitude, keeps constant throughout the storage season. It was the aim of the present investigation to test whether this was the case. The quantitative methods of estimating ethyl alcohol and acetaldehyde, which will be described in a later paper, have been employed to determine the CO_2 /alcohol number [see Fidler, 1933] ratio in selected varieties of apples placed under anaerobic conditions at different times during the storage season. The results for Bramley's Seedling and Newton Wonder apples are given in Section I, and the results of preliminary measurements of the CO_2 /alcohol number ratio in several other varieties of apples are shown in an appendix.

MATERIALS AND METHODS.

The apples used were from the population used throughout this work [Fidler, 1933]. They were placed in the apparatus already described [Fidler, 1933] at a temperature of 23° . The gas stream, after passing through the fruit chamber, bubbled through a wash-bottle containing sulphuric acid (as described in the first paper), and then through a Pettenkofer tube containing 60 cc. of standard baryta. These tubes were changed at intervals.

After the apples had attained the temperature of the bath, air was drawn through at a rapid rate for a period varying between 18 and 24 hours. The air-line thus being fixed, the air current was changed for pure nitrogen at the standard rate of 2 litres per hour¹.

The nitrogen used in the experiments was commercial cylinder nitrogen, and was found to contain between 0.5 and 1.2 % oxygen. This was removed by passing the gas over copper which was heated electrically to 650–700°. Gas analyses of the purified gas showed that this treatment reduced the percentage of oxygen to less than 0.05.

Fig. 1. Rates of CO₂-production (—), and rates of increase in the alcohol number (....) in Newton Wonder apples in nitrogen at 23°. Ordinate: g. CO₂ produced by 100 g. fruit during 100 hours or increase in alcohol number during 100 hours

Fig. 2. Rates of CO₂-production (—), and rates of increase in the alcohol number (....) in Bramley's Seedling apples in nitrogen at 23°. Ordinate: g. CO₂ produced by 100 g. fruit during 100 hours or increase in alcohol number during 100 hours.

At the end of the exposure to the gas (4 to 5 days), the fruit was removed from the apparatus, weighed, and the alcohol number was estimated. The CO₂ absorbed by the baryta was estimated by titrating the barium hydroxide-carbonate mixture against standard hydrochloric acid using phenolphthalein as an indicator.

Part of the same sample of fruit was analysed immediately on being received, and the alcohol number thus obtained was subtracted from that found in the above experiment, in order to get a measure of the effect of the absence of oxygen on zymasis.

¹ The rate of passage of the gas stream was measured in the first instance by collecting the emergent gas in a graduated jar over water. The rate of bubbling through a wash-bottle containing KOH (to remove CO₂ from gas entering the apparatus) was then noted. This rate of bubbling was reproduced in subsequent experiments.

Table I. CO_2 /alcohol number in apples in pure nitrogen at 23°.
(See Figs. 1 and 2.)

No. of exp.	Variety of fruit	State of fruit	Date exp. begins	Duration of exposure to nitrogen (hrs.)	No. of apples in sample	Wt. of sample (g.)		Per-centage CO_2 production (g./100 hrs.)	Increase in per-centage alcohol number during 100 hrs.	Ratio CO_2 /alcohol number (both expressed as gram-molecular equivalents)*	Glycolysis in C units/ CO_2 in C unit†
						Initial	Final				
1	2	3	4	5	6	7	8	9	10	11	12
Season 1929-30:											
Not plotted	1	Newton	1. iv. 30	121½	1	—	99.5	0.412	0.37	100:86	2.7
	3	Wonder	14. v. 30	123½	1	—	88.5	0.426	0.40	100:91	2.8
Season 1930-31:											
17	Newton	Green. Hard	24. ix. 30	97½	1	—	112.5	0.203	0.19	100:89	2.8
21	Wonder	Young. Hard	1. x. 30	100½	1	—	144.0	0.290	0.25	100:84	2.7
30		Green to green-yellow	27. xi. 30	97	1	—	193.0	0.242	0.21	100:84	2.7
37		Yellow, slightly wilted	8. i. 31	99½	1	—	105.0	0.338	0.35	100:99	3.0
Season 1931-32:											
73	Newton	Green. Hard. Very young	1. vii. 31	97½	12	103.0	96.5	0.887	0.70	100:75	2.5
79	Wonder	"	17. vii. 31	95	9	174.5	167.0	0.813	0.73	100:86	2.7
81		"	26. viii. 31	98½	3	208.0	201.0	0.448	0.38	100:81	2.6
95		Green	28. x. 31	99	2	252.5	246.0	0.384	0.32	100:80	2.6
109		Green to yellow-green	10. xii. 31	98½	2	298.5	293.5	0.520	0.50	100:93	2.8
125		Green-yellow. First sign of wilting	27. i. 32	99	1	84.0	81.5	0.549	0.50	100:88	2.7

Season 1932-33:

143	Newton	Small and young	16. viii. 32	120	4	276.0	273.5	0.264	0.19	100:69	2.4
	Wonder										
Not plotted	I { a b c	Green	19. x. 32	119	2	280.0	254.5	0.254	0.15	100:56	2.1
		"	19. x. 32	119	2	306.0	301.5	0.276	0.18	100:60	2.2
		"	19. x. 32	119	2	296.0	291.5	0.316	0.20	100:66	2.4
	III { a b c	"	10. xi. 32	95½	2	226.5	225.5	0.343	0.24	100:68	2.4
		"	10. xi. 32	95½	2	226.0	222.0	0.315	0.24	100:74	2.5
		"	10. xi. 32	95½	2	235.0	230.5	0.329	0.24	100:70	2.4

Season 1930-31:

25	Bramley's	Green. Hard. Young	19. xi. 30	97½	1	—	148.5	0.356	0.34	100:91	2.8
34	Seedling	Green. Firm	10. xii. 30	97	1	—	124.5	0.377	0.34	100:88	2.7
43		Green to green-yellow. Slight wilting and super- ficial scald	22. i. 31	98½	1	—	134.5	0.379	0.39	100:97	3.0

Season 1931-32:

72	Bramley's	Green. Hard. Very young	1. vii. 31	97½	11	238.5	233.0	0.714	0.43	100:58	2.2
78	Seedling	"	17. vii. 31	95	5	200.0	192.0	0.667	0.60	100:93	2.7
85		"	2. ix. 31	99	2	272.5	271.5	0.455	0.40	100:83	2.6
102		Very green	11. xi. 31	98½	1	223.0	218.0	0.592	0.50	100:80	2.6
117		Green	12. i. 32	99	1	187.5	185.5	0.456	0.32	100:67	2.4
134		Green-yellow	6. iii. 32	97½	1	138.0	134.5	0.631	0.50	100:76	2.6

Season 1932-33:

142	Bramley's	Small and young	16. viii. 32	120	4	247.0	247.0	0.252	0.25	100:94	2.9
	Seedling										
Not plotted	III { a b c	Green	24. xi. 32	95½	2	260.5	257.5	0.408	0.36	100:85	2.7
		"	24. xi. 32	95½	2	265.0	260.5	0.362	0.33	100:86	2.7
		"	24. xi. 32	95½	2	262.5	257.5	0.396	0.36	100:92	2.8

* The ratios given in column 11 are for the percentage CO₂ produced in unit time, and expressed as molecular equivalents/the percentage increase in the alcohol number in unit time, also expressed as molecular equivalents. It is of interest to note that the ratio ethyl alcohol/acetaldehyde was never less than 75/1. Since the present research is concerned only with the alcohol number and was not undertaken to determine this ratio, the alcohol numbers in the tables are not divided between ethyl alcohol and acetaldehyde.

† Blackman [1928] prefers to summarise the reaction as a quotient, glycolysis in carbon units/N.R. in carbon units. A column has been added (12) to permit of comparison with his results, should this be desired later. Each figure in this column represents the sum of the carbon equivalents of CO₂ (column 9) and of ethyl alcohol (column 10) divided by the carbon equivalent of CO₂ (column 9). No further mention will be made of this column here.

RESULTS AND CONCLUSIONS.

From the data for the alcohol number in column 10 of Table I, and the graphs of these data in Figs. 1 and 2, we see that in both Bramley's Seedling and Newton Wonder apples the average rate of anaerobic zymasis over a period of 100 hours appears to fall sharply from the value for samples of growing apples in late July to that for fully grown green apples. These changes for growing apples will be further investigated in the future. Our results for fully grown stored apples suggest that during the early stage of storage the rate of anaerobic zymasis changes but little. When, however, the colour change from green to yellow sets in, the rate of anaerobic zymasis appears to be accelerated.

Apparently the CO_2 output of these two varieties under anaerobic conditions fluctuates seasonally in a similar fashion (column 9, Table I, and Figs. 1 and 2). Thus the average rate of CO_2 output during a period of 100 hours of anaerobiosis falls sharply during the later phases of growth to a value which does not change significantly during the early phases of storage. The graphs suggest that the rate of CO_2 output by apples under anaerobic conditions increases as they turn yellow. If a connection is assumed to exist between anaerobic and aerobic processes, this rise, and also the fall during late orchard life, is what would be expected from the published results of other workers for the CO_2 output of apples in air [see Hardy, 1933].

The most important deduction to be made from the present data is that the ratio $\text{CO}_2/\text{alcohol number}$ (Table I, column 11) does not alter significantly with the season in spite of these seasonal changes in the rates of anaerobic zymasis (Table I, column 10), and anaerobic CO_2 output (Table I, column 9). It appears, therefore, that these two magnitudes fluctuate proportionally, and this conclusion is borne out by the parallelism of the courses followed by the graphs for the alcohol number and CO_2 -output in Figs. 1 and 2.

Table II. *Mean values for $\text{CO}_2/\text{alcohol number}$ in stored Newton Wonder and Bramley's Seedling apples.*

Variety	Season	Mean value of $\text{CO}_2/\text{alcohol}$ number	Deviation from mean	No. of measurements from which mean value is calculated
Newton Wonder	1929-30	100:88.5	± 2.5	2
	1930-31	100:89	$\begin{cases} +10 \\ -5 \end{cases}$	4
	1931-32	100:87	± 6	3
	1932-33	100:65.5	$\begin{cases} +8 \\ -5 \end{cases}$	6
	1929-30-31-32-33*	100:72.5	$\begin{cases} +27 \\ -16 \end{cases}$	15
Bramley's Seedling	1930-31	100:92	± 5	3
	1931-32	100:72	$\begin{cases} +8 \\ -5 \end{cases}$	3
	1932-33	100:87.5	$\begin{cases} +4.5 \\ -2.5 \end{cases}$	3
	1930-31-32-33*	100:84.5	$\begin{cases} +13 \\ -17 \end{cases}$	9

* See Introduction, and also Thomas and Fidler [1933].

This substantiates the important assumption implicit in Blackman's paper that the CO_2 /alcohol ratio remained constant during the period of storage in which his experiments were carried out. Considerable interest centres, therefore, in the mean value of this ratio for a given season.

The data in the above table show that, except in Newton Wonder apples in seasons 1929–32, there is a different mean value for the CO_2 /alcohol number ratio in each season. The difference in the mean value of this ratio for different seasons may be accidental, but experiments with large samples would be needed to settle this point.

From two observations on leaves of *Acer platanoides*, Kostytschew [1913] inferred that the ratio CO_2 /ethyl alcohol fluctuates with the stage of development of the tissue. But in the present experiments the fluctuations, though considerable, were not regular. They might be attributed to a combination of experimental error, the occurrence of physiological races within the varieties, and the use of small samples. Probably the last is the most important cause; for, as will be shown in a later paper, this sampling error is much larger than the maximum error encountered in the estimation of ethyl alcohol and acetaldehyde in apples.

The average value for the CO_2 /alcohol number ratio in Bramley's Seedling and Newton Wonder apples may be placed at 100/80–85. We have, however, obtained some evidence (see Appendix) of varietal differences in regard to the ratios.

As the CO_2 /alcohol number ratio in molecular equivalents is not unity, as demanded by the usual equation for alcoholic fermentation, $\text{C}_6\text{H}_{12}\text{O}_6 = 2\text{CO}_2 + 2\text{C}_2\text{H}_5\text{O}$, Blackman's method of evaluating glycolysis must be slightly modified. He assumes that the ratio is unity, and he calculates glycolysis from CO_2 output in nitrogen by multiplying this figure by 3. If, however, the CO_2 /alcohol number ratio is 100/85, only 1.7 atomic proportions of carbon would be lost as ethyl alcohol for each one lost as CO_2 , so that glycolysis would be 2.7 times the CO_2 output in nitrogen. In view of the fluctuations about a mean value of 100/85, the CO_2 /alcohol number ratio should be measured in a sample of the same fruit before attempting to estimate glycolysis. Many other examples are known of CO_2 /ethyl alcohol ratios in which CO_2 predominates, and only rarely has a ratio of unity been reported and confirmed. Thus Boysen-Jensen [1922] for grapes and carrots and Kostytschew [1913] for many tissues including apples obtained ratios ranging from 100/40 to 100/80. Both conclude that anaerobic respiration in higher plants is a mixed reaction which consists only in part of alcoholic fermentation, the accompanying reaction being independent of glycolysis.

This latter conclusion cannot be accepted unconditionally, for products of zymasic cleavage not estimated by the present method may account for the low ratios obtained. Acetaldehyde, however, cannot be one of these, since this compound is included in the alcohol number.

SUMMARY.

1. Zymasis in nitrogen follows the same seasonal curve as CO_2 output in air (O.R.).
2. In any one season, the ratio CO_2 /alcohol number (which is practically identical with the ratio CO_2 /ethyl alcohol) fluctuates widely about a mean value, but this mean does not alter as apples age.
3. The mean value for this ratio in Newton Wonder and Bramley's Seedling apples during three seasons is of the order 100/80–85.

4. The mean value for the CO_2 /alcohol number ratio may vary from season to season.

5. The results of preliminary experiments suggest that there are great varietal differences in the CO_2 /alcohol number ratio; this ratio fluctuates between 100/47 to 100/92 in small samples of different varieties of ripe apples.

In addition to the acknowledgments which have already been made [Fidler, 1933], I wish to thank the Armstrong College Research Committee for a grant for the purchase of nitrogen.

APPENDIX.

A SURVEY OF CO_2 /ALCOHOL NUMBER IN SEVERAL VARIETIES OF APPLES.

The following is an account of a short survey of CO_2 /alcohol number in several varieties of ripe apples purchased from local fruiterers. Estimations were carried out precisely as described in the main paper.

Variety	Country of origin	Date of commencement of exp.	Duration of exposure to nitrogen (hours)	g. CO_2 per 100 g. fruit per 100 hours	Increase in the % alcohol number in 100 hours (corrected for control values)	CO_2 /alcohol number
Dougherty	Australia	23. viii. 32	120	0.169	0.14	100 : 81
Gravenstein	U.S.A.	23. viii. 32	120	0.277	0.19	100 : 65
Sturmer	Australia	23. viii. 32	120	0.327	0.26	100 : 77
Keswick	England	1. ix. 32	120	0.264	0.16	100 : 57
Beauty of Bath	England	1. ix. 32	120	0.209	0.13	100 : 61
Bellflower	France	1. ix. 32	120	0.212	0.15	100 : 68
Maiden's Blush	U.S.A.	28. ix. 32	119½	0.289	0.22	100 : 73
Wealthy	U.S.A.	28. ix. 32	119½	0.167	0.08	100 : 47
Jonathan	U.S.A.	11. x. 32	120½	0.151	0.08	100 : 54
McIntosh Red	Canada	11. x. 32	120½	0.193	0.17	100 : 85
Cleopatra	Tasmania	11. vii. 30	99½	0.164	0.16	100 : 93
Cleopatra	Tasmania	6. v. 31	98½	0.255	0.21	100 : 85
Newtown Pippin	U.S.A.	8. iii. 33	114½	0.113	0.10	100 : 90
Newtown Pippin	U.S.A.	25. iv. 33	148½	0.177	0.15	100 : 81
Granny Smith	Australia	9. vi. 33	49	0.278	0.20	100 : 65

It appears that as for Bramley's Seedling and Newton Wonder apples, so also for these other varieties, the ratio CO_2 /alcohol number is always higher than that required by the usual equation for alcoholic fermentation. As small samples were used, the ratios given in the table cannot be taken as true means, but the differences shown suggest that the mean ratios may possibly vary greatly in different varieties of apples.

REFERENCES.

- Blackman (1928). *Proc. Roy. Soc. Lond.* B **106**, 446.
 Boysen-Jensen (1922). *Det. Kgl. Danske, Vidensk. Selsk. Biol. Meddel.* **4**, 1.
 Fidler (1933). *Biochem. J.* **27**, 1614.
 Hardy (1933). *J. Soc. Chem. Ind.* **52**, 46.
 Kostytschew (1913). *Ber. deutsch. bot. Ges.* **21**, 125.
 — (1927). *Plant respiration.* (P. Blakiston's Sons, Philadelphia.)
 Thomas and Fidler (1933). *Biochem. J.* **27**, 1629.