Respiratory Metabolism in Detached Rhododendron Leaves[1, 2]

D. T. Bourne and S. L. Ranson

Department of Botany, University of Newcastle upon Tyne, Newcastle upon Tyne, England

Some of the measurements described below were first made many years ago (21; see 26, 27, 28) as part of an investigation of the occurrence of the Pasteur effect in plant organs. More recently, with the development of interest in factors controlling respiratory metabolism, our attention was redirected to 2 of the plant materials originally studied, namely rhododendron leaves for which there was evidence of a marked Pasteur effect and young seedlings of buckwheat, the one material used for which no evidence of a Pasteur effect emerged. This paper describes 1 part of this work and is restricted to results obtained with detached whole leaves of rhododendron. Many investigations made for convenience with leaf discs or slices will be described elsewhere later, for their relevance to metabolism in the intact leaf is still in some doubt. The experiments described here are largely concerned with production and consumption of metabolites in the leaves in air, in pure nitrogen, and on return to air after a period in nitrogen. Particular attention has been directed toward transitory changes in concentrations of glycolytic intermediates and keto-acids as guides to changes of rates of glycolysis and of oxidative reactions of the tricarboxylic acid cycle. These data have the limitation of all such measurements in that they represent average concentrations for whole cells which may bear little relation to changes in specific metabolic pools associated with glycolysis and cycle oxidations but are nevertheless a necessary preliminary to further work. Also, because of renewed interest elsewhere in the origin of the anaerobic CO_2 output (24) and of the possible consumption of ethanol (9, 10, 29), some attention is given here to these aspects of metabolism.

There is an extensive amount of literature on respiratory metabolism in plant organs, but this is now well documented in text books (4, 16, 25, 26) and chapters in encyclopedic works (14, 23) and no further review will be attempted here.

Materials and Methods

Leaves of *Rhododendron ponticum* were gathered from local sources, washed and dried, and then divided into random samples of equal fresh weight and stored in darkness.

Gaseous Exchange. In early work, CO_2 outputs were measured by the Pettenkofer method, the $Ba(OH)_2$ solution being titrated at 3-hour intervals. In more recent experiments, the CO_2 output and O_2 uptakes have been computed from analyses made in an open circuit system in which dry CO_2-free air or nitrogen was passed at a constant rate over the leaves, then, again after drying, through an infrared gas analyser (Grubb Parsons) and through a Beckman Model E2 oxygen analyser. The infrared gas analyser was connected to a chart recorder to give a continuous record of CO_2 output; the O_2 content of the gas was read from the Beckman instrument as required and plotted manually. To change the atmosphere around the leaves, the new gas was flushed through the instrument at a rapid rate until the O_2 analyzer recorded the O_2 composition of the entering gas (usually about 10 min). The gas stream was then slowed to the standard constant rate (40 ml/min). A further period of about 10 minutes was required for the establishment of equilibrium conditions so that the first valid records of CO_2 and O_2 exchange were obtained about 20 minutes after change to the new atmosphere. Some records were obtained for single leaves using standard manometric techniques.

Analyses of Metabolites. Samples of leaves (100–200 g) were removed at intervals for analyses. Ethanol and acetaldehyde were recovered by steam distillation, oxidized with acid dichromate to acetic acid and estimated by titration (12). Acetaldehyde was determined, using a portion of the steam distillate, by the method of Clausen (7). Though nonspecific, these techniques give very similar results for rhododendron leaves, to the estimation of ethanol with yeast alcohol dehydrogenase and the esimation of acetaldehyde by colorimetric techniques (6). Ethanol and acetaldehyde emerging in gas streams were trapped in concentrated H_2SO_4 and determined together by the oxidation procedure.

Water soluble carbohydrates (referred to later as sugars) were extracted by the method of Porter and Martin (20) and, after treatment of the extract with absolute ethanol and animal charcoal to remove interfering materials, estimated using the anthrone reagent (8).

Water-soluble glycosides (other than sugars) were estimated by measuring the increase in the anthrone reaction of the extract following hydrolysis with N-HCl for 30 minutes and treatment with charcoal.

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Starch, which remained in the cell debris after the extraction of sugars, was recovered by 3 successive extractions with 30 % perchloric acid and estimated using the anthrone reagent.

Organic acids were extracted by blending leaves in boiling 80 % ethanol and by further extraction with water. This extract was passed through cation (H-form) and anion (carbonate form) exchange resins and the acids recovered from the latter by elution with 3 N $(NH_4)_2CO_3$ solution. The eluate, concentrated by boiling and freeze-drying, was analyzed by gradient elution from silica gel columns using chloroform-tertiary amyl alcohol mixtures and collecting the effluent in 2 ml fractions (30). To avoid water-logging of the gel, the chloroform was only 80 % saturated with 0.5 N H_2SO_4 before mixing with the amyl alcohol.

Keto-acids were extracted with cold 5 % metaphosphoric acid from leaves powdered in liquid nitrogen and converted to the 2,4-dinitrophenylhydrazones by the method of Isherwood and Niavis (15). The hydrazones were separated by chromatography on thin layers of Kieselgel G with a solvent composed (v/v) of 13 parts petroleum ether (B.P. 60-80°) and 7 parts ethyl formate to which was added acetic acid to a concentration of 2 moles/liter (22). After development, zones were scraped from the plates, extracted with 0.2 M $NaHCO_3$ and the hydrazone content measured spectrophotometrically at 365 mμ.

Phosphate esters were extracted and assayed enzymatically by methods very similar to those of Barker et al. (1). The leaves were dropped into liquid nitrogen, powdered and extracted with trichloroacetic acid. The trichloroacetic acid was then removed with ether and, after neutralization and removal of inhibitory substances with charcoal (Norit N.K.), the various ester phosphates were assayed spectrophotometrically at 340 mμ after appropriate additions of enzymes, coenzymes and cofactors.

Feeding Experiment. Samples (10 g) of leaves were enclosed in large boiling tubes fitted with rubber bungs with their petioles inserted into small phials containing the C^{14} glucose solution (2.5 μc, 155 μg, in 1 ml water). CO_2-free NaOH solution was placed in the bottom of the tube to trap CO_2 evolved. One set of samples was fed in air, another immediately after transfer to pure nitrogen, and the third immediately after return to air after a 20-hour period in nitrogen. The sugar was injected by evacuation for 1 minute after which the tube was refilled with the appropriate CO_2-free gas. Respired CO_2 was recovered as $BaCO_3$, weighed, plated on aluminum planchets and the C^{14} content assayed by the scintillation technique. Corrections were made for background and self-absorption.

Reagents. Enzymes (including glucose-6 P and 6-phosphogluconic dehydrogenases, phosphoglucose isomerase, glyceraldehyde-3 P dehydrogenase, aldolase, lactic and ethanol dehydrogenases, pyruvic and 3-phosphoglyceric kinases), coenzymes, cofactors and substrates were obtained from Boehringer Corporation, London Ltd; Norit NK from Hopkin and Williams Ltd; Kieselgel G from Shandon Scientific Company, Ltd. Glucose-1-C^{14} and glucose-6-C^{14} were obtained from the Radiochemical Center, Amersham and adjusted to a common specific activity (2.9 mc/mmole) before use.

Results

Gaseous Exchange. Detached mature leaves of rhododendron in darkness and in air at temperatures around 20° evolved CO_2 for periods extending to weeks without yellowing, browning, or other visible injury. The respiratory activity in terms of fresh weight decreased as the leaves aged on the bush, and was in general much higher in summer than winter for comparable leaves under comparable conditions. Immediately after collection and sorting, similar samples of leaves showed high but varied CO_2 outputs. In the course of the first day the CO_2 outputs decreased relatively quickly to a more static level and became more uniform. Respiratory quotients (RQ's) at or near unity at the outset decreased, often within the first day except in the summer months, to values approaching 0.8 at which they then remained for long periods. In part, at least the varied CO_2 outputs at the outset can be attributed to handling (11) and in some experiments, where numbers of comparable samples were required, they were maintained in darkness in air for several days before transfer to other environmental conditions. By this time RQ's were between 0.8 and 0.9, and the CO_2 outputs were well below their initial levels and decreasing relatively slowly. Such leaves are referred to subsequently as starved leaves.

At all times of the year the leaves showed markedly enhanced CO_2 outputs following transfer from air to nitrogen, i.e. to anaerobic conditions, and again on return to air after periods in nitrogen. The magnitude and duration of the increases following the transfers were much more marked for starved leaves than for relatively fresh leaves. In general, when leaves were respiring at high rates in air before transfer to nitrogen, the increases were much less marked than when leaves showed low respiratory activities at the outset. Figure 1 shows a typical response for leaves in the latter category. After some 80 hours in air the CO_2 output of the leaves was in the phase of slow decline and the RQ steady at about 0.8. In nitrogen the CO_2 output rose to a maximum then rapidly decreased again within 6 to 9 hours. Later the decrease was slower but the CO_2 output remained above the air line value, i.e. the comparable rate for leaves maintained in air, for the remainder of the 24-hour period in nitrogen. Estimation of the anaerobic CO_2 output (I) at the moment of transfer from air to nitrogen by extrapolation from the CO_2 output record (5) suggests that the transfer resulted in an increase of 80 % above the aerobic CO_2 output (N) in air at that time. Similar analyses of several comparable experiments gave I/N quotients between 1.9 and 2.2. Comparing meas-

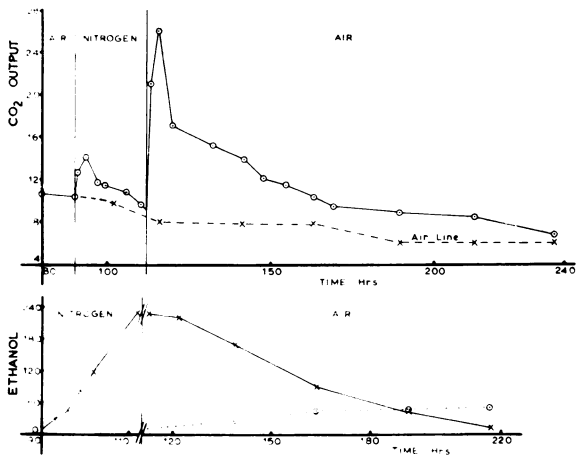

FIG. 1. (*Upper*). Time course of CO_2 outputs (mg/100 g fr wt/hr) from leaves in air, in nitrogen, and on return to air after 24 hours in nitrogen (⊙——⊙), and for leaves maintained throughout in air (× - - - ×). (*Lower*) Time course of ethanol (mg/100 g fr wt) accumulation and depletion in leaves in nitrogen and on return to air (×——×) and ethanol recovered from air stream (⊙ - - - ⊙). Time scale in hours after collection of leaves.

ured CO_2 outputs in air and nitrogen over various time intervals, the minimum I/N quotient recorded for a 24-hour period was 1.2.

On return to air, the CO_2 output increased even more rapidly to a higher maximum value in 3 to 6 hours but decreased equally rapidly to a lower rate within a further 3 hours (phase 1) and then slowly over a period of days (phase 2) towards the air-line value. A similar response was obtained in every case with starved leaves when the previous period in nitrogen was 24 hours or more. None of the leaves showed injury at the end of these long experiments.

In some experiments, in the summer months leaves were transferred to nitrogen within 24 hours of collection when the CO_2 output was still high and the RQ near unity. The rise in CO_2 output on transfer was less marked (fig 2) giving an I/N quotient for the moment of transfer of about 1.2. After the first rise the anaerobic CO_2 output declined until after 24 hours it was less than the air-line value. The I/N quotient for the 24-hour period was less than unity in this instance. On return to air the CO_2 output increased rapidly to a value above the air-line value, but the initial response was much less marked than for the starved leaves.

CO_2 outputs in excess of the air-line values were observed on return to air after periods of 90 minutes in nitrogen, and a series of records for comparable leaves suggests that the magnitude of the initial increase on return to air increased with the duration of the previous period in nitrogen up to 12 hours. With further extension of the anaerobic period the effects were less clear cut.

Because of the method of measurement, O_2 uptakes within the first 20 minutes after return to air remained uncertain but it is clear that within 30 minutes they reached their maximum values and thereafter decreased (cf. fig 2). This post-anaerobic O_2 uptake reached its maximum well before that of the CO_2 output and in the 20- to 30-minute period after transfer the RQ's were very low. In the experiment recorded the RQ rose within the first hour following the return to air to a value near unity, that is to the value recorded in air before the transfer to nitrogen.

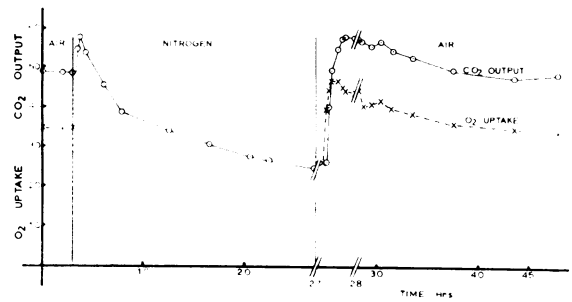

FIG. 2. O_2 uptake (× - - - - ×) and CO_2 output (⊙——⊙) of leaves (mg/100 g fr wt/hr) in air, on transfer to nitrogen and on return to air. Time scale in hours after collection of leaves.

Other experiments yielded similar results to the extent that within about 1 hour after the return to air the RQ's rose rapidly to values close to those recorded before the transfer to nitrogen whether these were nearer 0.8 or unity.

Ethanol Accumulation and Depletion. In mature leaves under anaerobic conditions ethanol accumulated steadily though at decreasing rates; the longest period investigated was 3 days at 20° when the final accumulation exceeded 500 mg/100 g fresh weight. In the experiment with starved leaves (fig 1) the estimated rate of ethanol accumulation immediately after transfer to nitrogen was 11.7 mg/hour as compared with an initial rate of CO_2 output (extrapolated value) of 20 mg/hour so yielding a molar ethanol/CO_2 quotient of 0.55. Comparisons of the measured ethanol accumulations and CO_2 outputs over increasing time intervals gave quotients rising to 0.8 after 24 hours. In similar experiments with leaves at various stages of starvation, the ethanol/CO_2 quotient immediately after transfer to nitrogen, invariably lay between 0.55 and 0.6 and in most of these experiments very similar values were obtained when the comparisons were made for periods extending to 48 hours. It appears therefore that considerably more than 1 mole of CO_2 was released for each mole of ethanol accumulated during the first hours in nitrogen and, moreover, that the production of CO_2 in excess of ethanol persisted for long periods under anaerobiosis. The amount of acetaldehyde present never exceeded 1 % of the ethanol content. On return to air the ethanol disappeared from the leaves at a decreasing rate over a period of days. Figure 1 sug-

gests that immediately after the return to air the rate of utilization was slow but whether this is in fact the case is doubtful. Clarke (6) obtained data in 1 experiment with rhododendron leaves which indicated that ethanol continued to accumulate for about 3 hours after the leaves were returned to air from nitrogen and then immediately decreased again. In the experiments described here the first ethanol measurements were made some hours after return to air so that the phase of accumulation in air would be missed, if it occurred. In every experiment the overall rate of depletion was less than the rate of accumulation; the maximum rate of depletion was on no occasion greater than 40 % of the maximum rate of accumulation in the same experiment. Some ethanol escaped from the leaves in the air stream but the amount detected in the H_2SO_4 traps never accounted for more than 25 % of that lost from the leaves and it is inferred that most of the ethanol was consumed in the leaves. Clarke (6) observed a temporary increase in acetaldehyde on return to air but at its maximum level it represented only a few percent of the ethanol content. In general (cf. fig 1) the CO_2 output of the leaves returned to the air-line value at about the same time as the ethanol disappeared.

Relative Rates of Substrate Utilization in Air and Nitrogen. In the starved leaves (fig 1) the quotient, anaerobic carbon loss in CO_2 and ethanol/aerobic carbon loss in CO_2, when compiled from estimated rates for the moment of transfer from air into nitrogen, was about 4. When calculated from measured amounts of CO_2 evolved and ethanol accumulated over a 24-hour period the value was 3. In other experiments with leaves at a similar stage, the maximum value was about 7 for the moment of transfer and the minimum 2.6 measured over a period of 48 hours. For fresh leaves (fig 2) the values for the quotient were much lower, namely 2.4 at the time of transfer and 1.5 for the 24-hour period in nitrogen. These

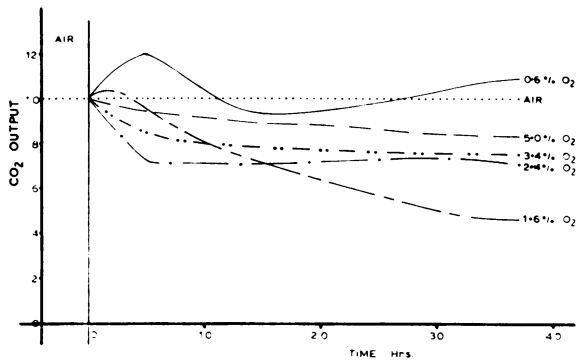

FIG. 3. Relative CO_2 outputs of leaves in air, 0.6 %, 1.6 %, 2.4 %, 3.4 % and 5 % O_2.

figures suggest that the presence of O_2 exerts a conserving effect on respiratory substrate in the leaves and that the conservation was more marked in the starved leaves than the fresher leaves.

CO_2 Evolution and Ethanol Accumulation in Atmospheres Containing Low O_2 Concentrations. The results of representative experiments are summarized in figure 3. These measurements in different O_2 concentrations were made at different times on different samples of leaves with CO_2 output rates in air at the time of transfer to the new atmosphere varying between 5 and 10 mg/100 g fresh weight per hour, but they are here shown for brevity, against a common air rate of 10 mg/hour. In 0.6 % O_2 there was a defined though slight increase in CO_2 output for a few hours after which it declined and remained not very much different from that in air. Ethanol accumulated slowly and steadily throughout a 50-hour period in 0.6 % O_2 and ethanol/CO_2 quotients for the period, however computed, lay between 0.1 and 0.2. In 1.6 % oxygen after a transitory and ill-defined increase in CO_2 output lasting only 2 to 3 hours the CO_2 output fell markedly below that in air. There

Table I. *Carbohydrate Content and CO_2 Output; May*

Starch, sugar, and glycoside contents (as mg glucose/100 g fr wt), CO_2 outputs (mg/100 g fr wt/hr) and RQ's of detached leaves stored in darkness. Temperature 25°.

Time (hr)	Starch	Sugar	Sugar + starch	Glycosides	CO_2 output	RQ
		(in air)				
12	460	1430	1890	1850	43	0.96
32	560	970	1530	2070	41	0.91
56	700	890	1590	2240	37	0.97
81	675	950	1625	2230	38	0.96
105	560	950	1510	1940	41	0.96
137	440	1050	1490	1750	...	...
158	470	1040	1510	1770	38	0.99
		In nitrogen (transferred from air after 12 hrs)				
Start	460	1430	1890	1850		
25	350	880	1230	2100		
		In air (transferred from nitrogen after 25 hrs)				
Start	350	880	1230	2100		
22	230	1180	1410	2000		

was some slight indication of ethanol accumulation (5 mg/100 g fr wt) in the first few hours but thereafter none. On transfer from air to 2.4 %, 3.4 % and 5 % O_2 the CO_2 outputs decreased without any preliminary rise and there were no detectable accumulations of ethanol. The conclusion is that some O_2 concentration below 2.4 % but above 1.6 % was adequate to sustain a completely aerobic metabolism.

Carbohydrate Utilization in Air and Nitrogen. The changes in levels of soluble sugars and starch in leaves collected in May and maintained in the dark in air for several days are shown in table I. The main loss in sugar occurred on the first day and thereafter changes were slight. Starch appeared to increase for some days, then to decrease again, but over the whole period there was little net change. The interesting point is that after the first day there was little change in total measured carbohydrates although the CO_2 output continued at only a slowly decreasing rate. Moreover the RQ remained virtually unchanged. Even for the first 20 hours the loss of 360 mg of carbohydrate would account for less than 70 % of the observed CO_2 output. Later it would appear that carbohydrate contributed virtually nothing to the CO_2 evolved in air. In nitrogen the mean rate of depletion of carbohydrate was nearly 50 % greater than in air. Even so if we assume as found in other experiments that the carbon appearing in CO_2 and ethanol in nitrogen in 25 hours would be at least 50 % greater than that released as CO_2 in air, the decrease in the carbohydrate did not account for all of the CO_2 and ethanol produced. Moreover, as shown earlier the CO_2 output is markedly enhanced on return to air from nitrogen but in these leaves there was no apparent loss of carbohydrate on return to air. In fact the change recorded was an increase. Clearly some carbohydrate reserve other than the starch or sugars extracted in these experiments could be the source of CO_2. There appeared to be no major change however in the amount of sugar bound in the water-soluble glycoside fraction.

Essentially similar results were obtained for leaves in the winter months when the CO_2 output in air was about 20 mg per hour. The starch of the leaves did not at any time exceed 5 mg/100 g fresh weight and therefore can be ignored. The sugar loss in air over the first 64 hours was equivalent to less than 50 % of the CO_2 output (table II). For the first 16 hours in nitrogen the rate of sugar loss was more than 4 times the maximum rate of loss in air and therefore probably great enough to account for the anaerobic CO_2 and ethanol production in that period. Later the rate of sugar utilization decreased and after 41.5 hours in nitrogen apparently ceased completely though CO_2 and ethanol production continued. Again on return to air the change recorded appeared to be some increase in carbohydrate content, not the depletion required to account for the rapid rate of CO_2 output known to occur at this time.

One interesting feature in both of these experiments is that the consumption of sugars appeared to cease before the total contents of these in the leaves were consumed and while CO_2 evolution continued actively.

Organic Acids. The plant acids present were malic, isocitric, and citric together with smaller amounts of succinate and fumarate (table III) but taken together the overall concentration was low, viz. about 8 mM. On prolonged starvation in air no overall depletion was observed nor was there evidence of rapid depletion of the acids as a whole after transfer from air into nitrogen or after transfer from nitrogen into air. It is clear, therefore, that the consumption of reserves of these acids at no time made any major contribution to the CO_2 evolved. Any changes in levels of individual acids shown in table III which are less than about 15 % are of doubtful significance. There is no doubt, however, that succinate accumulated throughout the first 12 hours in nitrogen and that on return to air it was very quickly consumed. Lactate, similarly, accumulated in nitrogen and then later was rapidly consumed in air. However, the maximum lactate accumulation would,

Table II. *Soluble Carbohydrate Content; January*

Soluble carbohydrate as mg glucose/100 g fresh weight of detached leaves stored in darkness. Temperature 25°.

Atmosphere	Air	Nitrogen (transferred after 12 hrs in air)		Air (transferred after 87 hrs in nitrogen)	
Time (hr)	Glucose	Time (hr)	Glucose	Time (hr)	Glucose
12	1780	Start	1780	Start	640
29	1700	16	1040	10	840
54	1640	41	590	24	910
77	1340	64	600	50	880
91	1370	87	640	74	870
124	1450	111	770	99	810
148	1150	135	730		
196	1400	184	610		

Table III. *Organic Acid Content; May*

Organic acid contents (μmoles per 100 g fr wt) of detached leaves stored in darkness.. Temperature 25°.

Time (hr)	Fumaric	Lactic	Succinic	Malic	Citric	Isocitric	Total cycle acids
		In air					
12	12	3.6	24	249	82	335	701
37	11	3.3	28	223	91	437	789
61	12	...	24	241	80	437	795
86	13	3.0	29	301	122	378	842
		In nitrogen (transferred after 12 hrs in air)					
Start	12	3.6	24	249	82	335	701
0.75	12	10.3	48	285	134	327	806
1.5	15	9.1	64	199	128	406	812
3	16	9.4	74	214	102	278	684
6	12	25.5	78	235	122	233	682
12	11	27.6	101	205	86	213	616
24	13	17.0	97	241	103	284	738
48	12	15.2	103	278	96	277	766
72	9	13.3	117	147	120	239	632
		In air after a period of 24 hrs in nitrogen					
Start	13	17.0	97	241	103	284	738
0.75	11	4.1	48	205	127	389	780
1.5	12	3.6	45	252	134	412	857
3	8	2.4	20	178	97	307	611
6	15	6.1	34	258	131	297	736
12	12	4.8	19	241	116	303	693
25	15	7.2	39	302	155	365	876
49	29	7.6	35	286	140	374	864

Table IV. *Keto-Acid Contents; April*

Keto-acid contents (μmoles per 100 g fr wt) of detached leaves stored in darkness. Temperature 25°.

Time	Pyruvate	α-Ketoglutarate	Oxaloacetate	Glyoxylate
		In air		
12 hr	44	200	16.5	5.2
36 hr	34	177	12.7	5.7
60 hr	30	137	8.3	6.0
		In nitrogen (transferred after 12 hrs in air)		
Start	44	200	16.5	5.2
5 min	33	186	12.0	3.5
15 min	60	221	10.0	4.2
30 min	103	239	8.5	4.2
1 hr	89	123	6.3	4.2
2 hr	88	122	6.2	4.0
4 hr	75	73	4.3	5.2
10 hr	70	50	3.5	7.2
24 hr	64	36	2.5	9.7
48 hr	45	14	1.0	11.0
		In air after a period of 24 hrs in nitrogen		
Start	64	36	2.5	9.7
15 min	58	41	3.7	8.0
30 min	47	42	3.7	8.2
1 hr	39	65	4.2	8.2
2 hr	37	80	6.5	6.5
4 hr	44	109	8.0	9.2
10 hr	40	129	8.0	7.7
24 hr	41	162	9.0	6.2

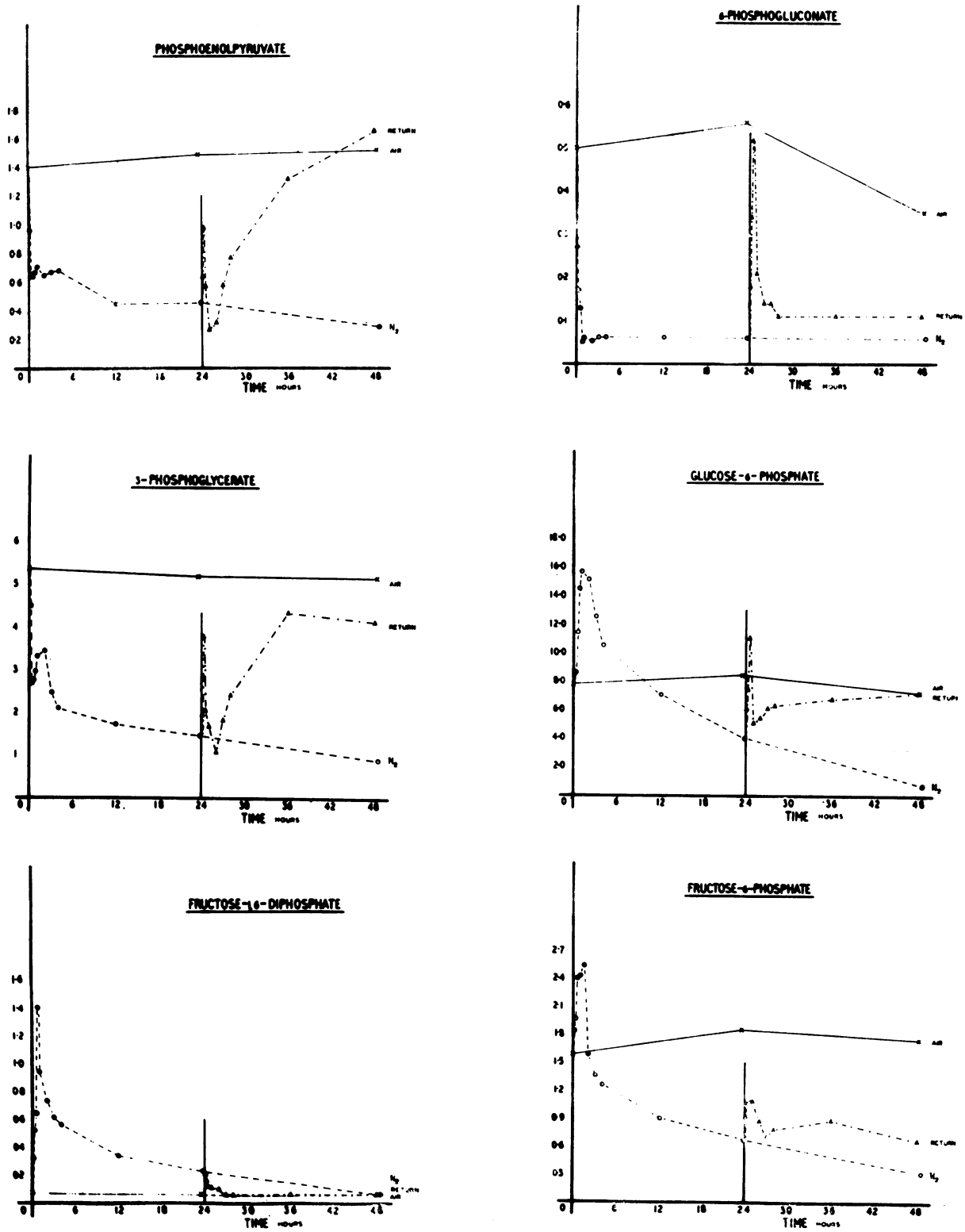

FIG. 4. Concentrations (μmoles/100 g fr wt) of phosphorylated acids and sugars in leaves maintained in air (×——×), transferred to nitrogen (○- - -○), and transferred to air after 24 hour in nitrogen (△-.-.△). Time scales start 12 hours after collection.

on a molar basis, represent less than 0.5 % of the ethanol accumulated. Some significance may attach to the suggestion of transitory changes in tricarboxylic acids and malic acids on transfer into nitrogen and again on return into air.

Keto-Acids. Pyruvic, oxaloacetic, α-ketoglutaric, and glyoxylic acids were all detected in the leaves, the α-ketoglutaric at about the same concentration as malic and citric acids. The levels of all of the keto-acids with the exception of glyoxylic appeared to decline rather slowly on starvation in air (table IV).

On transfer to nitrogen the concentration of oxaloacetate dropped to one-half within 1 hour and thereafter more slowly to a very low level. On return to air it returned slowly in the course of 24 hours to the air value. Pyruvate, after a possible slight decrease in the first 5 minutes, showed a 3-fold increase within 30 minutes. Later it decreased slowly but even after nearly 2 days in nitrogen the level remained higher than in air. α-Ketoglutarate showed a slight accumulation in the first 30 minutes but later it was rapidly depleted to a level about 10 % of the corresponding air value. On return to air both pyruvate and α-ketoglutarate returned slowly to their normal air values in the course of 24 hours. Apart from a suggestion of a temporary decrease within 5 minutes of transfer to nitrogen the changes in level of glyoxylate were slow, possibly indicating that the changes into and out of nitrogen had little direct effect on its metabolism.

Ester Phosphates. The levels of glucose-6-P, fructose-6-P, fructose-1,6-diP, 3-P-glycerate, P-enolpyruvate and 6-P-gluconate remained fairly constant over a 48-hour period in the dark in air (fig 4). On transfer to nitrogen there were very rapid decreases in the concentrations of 3-P-glycerate, P-enolpyruvate, and 6-P-gluconate but all of the hexose phosphates showed marked increases within an hour and then decreased again. On return to air after 24 hours in nitrogen there were transient but marked increases in the level of all but fructose-1,6-diP, and all except 6-P-gluconate and fructose-6-P had returned to something like normal values for air within about 12 hours. The combined total content of all of these phosphates at no time exceeded about 20 μmoles so that as consumable respiratory substrates they can be neglected. However, the transient changes of levels recorded on transfer from air to nitrogen and vice versa may, as mentioned later, have significance in portraying flux changes through the glycolytic and related pathways of metabolism.

$C^{14}O_2$ Yields from Glucose-1-C^{14} and -6-C^{14}. The barium carbonate recoveries (table V) indicate reasonable agreement between samples and a steady CO_2 output over the 24-hour period for the leaves in air. In nitrogen the CO_2 output followed a trend similar to that shown in figure 2, being slightly greater than for the leaves in air in the first 3 hours but less over the 24-hour period. For leaves on return to air after a period in nitrogen the CO_2 outputs

Table V. *Utilization of Glucose-1-C^{14} and Glucose-6-C^{14}*

CO_2 output (as mg $BaCO_3$ recovered) and $C^{14}O_2$ yield (counts/sec) from 10 g samples of leaves supplied in darkness with either glucose-1-C^{14} of glucose-6-C^{14} (a) in air, (b) in nitrogen and (c) in air immediately after a 20-hour period in nitrogen, and the quotient, C_6/C_1.

Time period	Glucose supplied	CO_2 output	$C^{14}O_2$ released	C_6/C_1*
		(a) In air		
1 hr	1-C^{14}	4.0	69	0.4
	6-C^{14}	4.5	34	
3 hr	1-C^{14}	12.0	1300	0.3
	6-C^{14}	12.1	380	
24 hr	1-C^{14}	101	8800	0.6
	6-C^{14}	95	5100	
		(b) In nitrogen		
1 hr	1-C^{14}	4.3	83	0.2
	6-C^{14}	4.4	18	
3 hr	1-C^{14}	14.5	760	0.2
	6-C^{14}	13.8	117	
24 hr	1-C^{14}	79	8600	0.4
	6-C^{14}	90	3700	
		(c) In air after return from nitrogen		
1 hr	1-C^{14}	9.5	10	0.8
	6-C^{14}	9.1	8	
3 hr	1-C^{14}	25	89	0.4
	6-C^{14}	32	38	
24 hr	1-C^{14}	196	8300	0.3
	6-C^{14}	202	2800	

* $C^{14}O_2$ yield from glucose-6-C^{14}/$C^{14}O_2$ yield from glucose-1-C^{14}.

were, as to be expected, much in excess of those of the leaves retained in air after 1 hour and remained so after 24 hours.

In air it is clear that for the glucose supplied to the leaves carbon atom 1 was released as CO_2 more rapidly than carbon atom 6 which suggests that the glucose supplied was consumed at least partially in reactions other than glycolysis, and possibly the participation of the pentose phosphate sequence in CO_2 release. Under anaerobic conditions if the CO_2 liberated is produced wholly from sugar in fermentative reactions no labeled CO_2 would be liberated from either C_1 or C_6 labeled glucose. In this experiment the yield of $C^{14}O_2$ from glucose-6-C^{14} was less than in air throughout the 24-hour period but it was nevertheless appreciable. This suggests that there was considerable sugar turnover in reactions other than those of glycolysis and considerable randomization of the label in hexose sugars as a result. Alternatively it must be supposed that the reactions of the TCA cycle continued in some way in the absence of O_2 or that the system was not completely anaerobic. The measured CO_2 output is not inconsistent with the presence of a low percentage of O_2 (cf. fig 3). Possibly however, the $C^{14}O_2$ yields particularly for the first hours in nitrogen may be taken to indicate that the conversion of C_1 of glucose to CO_2, relative to that of C_6, was enhanced. One possible explanation is that the enhanced carbohydrate consumption which occurs on transfer to anaerobic conditions involves a greater participation of the pentose phosphate sequence and that this yields, eventually, pyruvate which is available for conversion into ethanol with the release of more CO_2.

The data for leaves on return to air after a period in nitrogen are possibly the most interesting in that they indicate that though the CO_2 output was markedly enhanced the labeled sugars presented to the leaves contributed very little to this CO_2 in the first few hours. This could be a result of a slower penetration of the labeled sugars, after a period of anaerobiosis, to appropriate sites in the cells or alternatively to preferential consumption of substrates other than sugars. The fact that after 24 hours the yields of $C^{14}O_2$ from the sugars were more nearly comparable with those measured for leaves retained in air suggests that the latter alternative may be the more nearly correct.

Discussion

Respiration in Air. The analyses of carbohydrates present in the detached leaves suggest that after the first 12 hours for which no data are available, carbohydrate utilization accounts for only a part of the CO_2 evolved and in later phases of starvation for very little if any of it. Carbohydrate utilization appeared to stop when there were appreciable amounts remaining in the leaves and the presumption is that either this carbohydrate occurred in a form, or in pools, not readily accessible to the respiratory enzymes or that some other substrate became available after a time and then was preferentially consumed. No evidence was obtained that sugars present in glycosidic combinations were utilized to any extent during starvation but it remains possible that some component carbohydrate, not measured by the techniques used, served as a respiratory substrate. In most of the experiments it is unlikely that some undetermined carbohydrate supplied the bulk of the CO_2, for the RQ's drifted to values about 0.8. However in 1 case (table 1) the RQ's appeared to remain near unity for some days though the carbohydrates measured showed little change. Nonvolatile di- and tri-carboxylic acids taken together were not present in a quantity which alone would sustain the CO_2 output for more than a few hours and even after several days in darkness there was no marked overall depletion of acid. There are no obvious fatty reserves in the leaves and, as others have concluded from experiments with detached leaves (see e.g. 18), it appears probable that after a relatively short period of starvation, CO_2 is derived in increasing proportion from turnover in the nitrogenous components of the leaves.

Anaerobic Respiration. In the absence of O_2 the CO_2 output was enhanced, at least for a period, and ethanol accumulated. Respiratory activity appeared to be at a minimum in the presence of about 2 % O_2 when ethanol accumulation was suppressed and the CO_2 output declined below the air-line value without the initial enhancement observed at lower tensions of O_2 where anaerobic metabolism was not completely suppressed.

In anaerobiosis the ethanol accumulation increased as the CO_2 output continued but the molar quotient for ethanol accumulation/CO_2 evolved remained fairly constant at values near 0.6 throughout long periods. Some acetaldehyde and lactate accumulated simultaneously but neither represented more than 1 % of the ethanol present. Hence, if the ethanol was produced in normal fermentative reactions from pyruvate with the release of an equimolar quantity of CO_2, either more ethanol was produced than actually accumulated or some extra CO_2 was produced in accompanying reactions. Ethanol/CO_2 quotients less than unity are of common occurrence in plant tissues but there are some which yield quotients near unity (see 4, 26, 27, 28) and, in the absence of evidence that ethanol can be consumed anaerobically, it is usually accepted that the lower quotients indicate the production of extra CO_2 in reactions other than those involved in the production of ethanol from pyruvate. For some tissues it appears that this extra CO_2 production is a transient phenomenon (see e.g. 24 for data on swede discs) which occurs for a limited period only following the transfer to anaerobic conditions and that thereafter CO_2 and ethanol are produced in equal amounts. In the detached rhododendron leaves, however, as in some other organs we have investigated (*Rumex* leaves, seedlings of *Cucurbita pepo*, and *Fagopyrum esculentum*) the rate of ethanol production remained less

than CO_2 production for long periods under anaerobic conditions.

The rate of substrate consumption estimated as carbon loss in CO_2 evolution and ethanol accumulation in nitrogen was greater than the carbon loss (as CO_2) in air. The difference was much more marked for leaves showing low CO_2 outputs in air after prolonged starvation than for leaves showing higher rates. The reason for this difference is not clear; it could reflect a difference in the substrates utilized in the 2 cases though there is no evidence to support this in data presently available. Another possibility is that the leaves showing high rates in air were nearer some inherent maximum than those showing low rates and therefore were less capable of further increase on transfer to anaerobic conditions. As pointed out by Thomas (26), however, comparisons of anaerobic rates of carbon loss with aerobic rates in air do not assess the full effect of O_2 in conserving substrate. Respiratory catabolism was at a minimum and completely aerobic at the extinction point, i.e. about 1.6 % O_2 for rhododendron leaves. Comparison of the anaerobic rates with the aerobic rates at 1.6 % O_2 would, therefore, show the full conservation effects and these would be greater than those assessed in the preceeding section, from rates in air and nitrogen.

The measured rates of carbohydrate depletion after transfer to nitrogen were greater than those in air. In 1 experiment (table I), the average rate for 25 hours in nitrogen was 50 % greater than the air rate but even assuming that the carbon loss was increased by only 50 % on transfer to the anaerobic atmosphere the observed carbohydrate depletion would account for little more than half of it. This estimate is based upon the assumption that the molar ethanol/CO_2 quotient would be 0.6 as observed in other experiments, but even if it were proposed that the ethanol together with an equimolar amount of CO_2 were produced from carbohydrate and the remainder of the CO_2 from another source, the observed carbohydrate depletion was only about 75 % of the required amount. In the other experiment quoted here (table II) for leaves in the winter months, the anaerobic sugar loss was for a period several-fold that in air and for this period, therefore, possibly adequate to sustain the anaerobic production of CO_2 and ethanol. Subsequently little further depletion of carbohydrate was detected though there is no doubt that CO_2 and ethanol production would continue at appreciable rates. It appears, therefore, that for these starving rhododendron leaves there is not only the question of the source of the extra CO_2 to be considered but also the source, at times, of considerable proportions of the total CO_2 and ethanol produced. The data presented here throw little light on these problems. For apple fruits Fidler (13) found that the loss in acid which occurred in air continued unchanged in the absence of O_2 and that the loss of carbohydrate plus acid accounted quantitatively for both the aerobic and anerobic productions of ethanol and CO_2. The organic acid fraction in rhododendron leaves showed no overall and sustained depletion either in nitrogen or air and it may be that, as for the leaves in air, turnover in nitrogenous components accounted for a considerable fraction of the ethanol and CO_2 production.

It has been suggested on other occasions (28) that decarboxylations of organic acids may contribute to CO_2 production, perhaps supplying the extra CO_2 component, on transfer of plant tissues to anaerobic conditions. In rhododendron it is clear that a fraction of the CO_2 may have arisen in this way. For example, α-ketoglutaric acid decreased by approximately 200 μmoles within 24 hours in nitrogen and if this represented consumption involving a single decarboxylation, the yield of 9 mg of CO_2 would constitute about 1 % of the total evolved in this period. The succinate which accumulated might possibly be produced in the course of these reactions. There is also in the data for acid contents some suggestion of an increase in total acids in the first 90 minutes of anaerobiosis followed later by a decrease. The decrease was attributable largely to a decrease in tricarboxylic acids, which might involve CO_2 release. In the absence of evidence about the way in which these acids may be consumed in the complete absence of O_2, it is not possible to estimate their contribution to CO_2 output with certainty, but if the observed decrease of about 200 μmoles represented the conversion, by reactions at present unknown, of tricarboxylic acids to CO_2, the yield would still represent less than 5 % of the total CO_2 evolved.

For swede discs where extra CO_2 is produced only for the first hour of anaerobiosis, Simon (24) has suggested the involvement of organic acids but in a different way. He obtained some evidence that the extra CO_2 in these discs came ultimately from carbohydrate reserves and involved an oxidative decarboxylation. He pointed out that this oxidation would of necessity be coupled with the reduction of some cellular components; the amount of CO_2 liberated ruled out the possibility that pyridine nucleotides were the final hydrogen acceptors and he suggested the possibility that acids might serve as the ultimate oxidants. Changes in acid levels recorded here for the first hour or so in nitrogen may reflect this function of acids but no simple interpretation along these lines can be attempted. Relevant also to Simon's observations is the rapid depletion of 6-P-gluconate observed in the leaves on transfer to nitrogen. If extra CO_2 is derived from carbohydrate at this stage, therefore, it may be that the oxidative decarboxylation occurs in reactions of the pentose phosphate cycles.

Pasteur Effect. Comparisons of relative rates of carbon loss in air and nitrogen suggested the occurrence of a Pasteur effect in rhododendron leaves and though it is clear that only a fraction of the carbon involved may have come from carbohydrate there is direct evidence of the occurrence of the effect in the data for carbohydrate contents which indicates that carbohydrate was utilized more rapidly anaerobically than aerobically. For the moment, we shall note

here only that the transient changes in levels of glycolytic intermediates on transfer from air to nitrogen were in essentials similar to those observed by Barker, Khan and Solomos (2) for pea seedlings which also show a marked Pasteur effect. Notably, there was a very rapid depletion of 3-P-glycerate and P-enolpyruvate and, slightly later, a rapid and marked increase in fructose-1,6-diP. The suggestion in the data for pea seedlings that glucose-6-P and fructose-6-P increase at first on transfer to nitrogen is amply substantiated in the rhododendron data where both showed marked initial increases and though declining later remained above the air-line values for some hours. The interpretation given by Barker et al. (2) to their data is based on the earlier suggestion (17, 19) that the rate of glycolysis is controlled by levels of ATP, ADP and Pi, but they have elaborated upon this and discussed their results in terms of a concept that glycolysis occurs within a glycolytic granule in cells. It is postulated that the bulk of the fructokinase, part of the phosphohexose isomerase and hexokinase, together with the other glycolytic enzymes are housed in this granule which is supposed to be permeable to ADP from the rest of the cell but much less permeable to ATP. Importance is attached to their observation that the depletion of 3-P-glycerate and P-enolpyruvate preceeded in time the increase in fructose-1,6-diP. They supposed that the onset of anaerobiosis may first activate the conversion of P-enolpyruvate into pyruvate, possibly because of increased availability of ADP, so producing ATP (protected in the granule from consumption in general cellular processes) which would then stimulate the first phosphorylative stages of glycolysis and hence increase the overall rate of glycolysis. In rhododendron the increase in pyruvate following the transfer to nitrogen reached its peak at about the same time as the P-enolpyruvate reached its minimum value and so is consistent with the hypothesis. However, until more information is available for the early stages of anaerobiosis about the changes in level of acetaldehyde ethanol and of other intermediates (e.g. α-glycerol-P) of reactions which may participate in the regeneration of NAD required for the conversion of triose-P into 3-P-glycerate, conjecture about other interpretations will be deferred.

One further point of note in the rhododendron data is the very rapid depletion of 6-P-gluconate on transfer to nitrogen. This could result from a marked retardation of its production as might be expected if it is produced oxidatively from glucose-6-P. Consistent with this view is the observed accumulation of glucose-6-P. Alternatively the depletion of 6-P-gluconate may result from an increased rate of consumption and again other observations are consistent with this view. For example, in the experiment with labeled glucose, CO_2 was released more rapidly from carbon atom 1 relative to that from carbon atom 6 in the first hour in nitrogen than it was over a similar period in air. If the dehydrogenases participating in the pentose phosphate cycle are spatially separated from enzymes of glycolysis, as may be the case if there is a glycolytic granule, any change in level of 6-P-gluconate might bear little relation to the change occurring in the total glucose-6-P content of the cell.

Respiratory Metabolism on Return to Air. Though the CO_2 output was markedly enhanced in this period the analyses of carbohydrates showed no signs of their rapid depletion. In fact in both sets of data reported here there are indications of some increase in sugar content following return to air. Again, also the total acid content showed no major change. The one substance present in appreciable quantity and observed to decrease in this period was the ethanol, accumulated during the preceding anaerobiosis. Indeed discounting the peak in CO_2 output in the first 3 to 6 hours on return to air (fig 1), the CO_2 output declined towards the air-line value roughly in parallel with ethanol content. The total CO_2 output of the post-anaerobic period was however greatly in excess of the possible production from ethanol consumed. In the experiment illustrated (fig 1) the ethanol consumption was 164 mg which would yield a maximum of 315 mg of CO_2. The total CO_2 output was about 1300 mg i.e. about 670 mg more than that recorded for leaves retained in air. Discounting the peak of the CO_2 output in the first 6 hours, the excess CO_2 output (500 mg) was still greater than that to be derived from the ethanol. Slightly better agreement was obtained in similar analyses of other experiments. As a working hypothesis therefore, it may be supposed that the total CO_2 output is composed of at least 3 components, namely a CO_2 output derived from ethanol consumed, superimposed on a ground respiratory CO_2 output, and supplemented by a CO_2 output from a third source particularly in the first few hours after return to air.

The substrate used in the ground CO_2 production was not detected in the present study but the RQ of leaves after the first hour or so in air returned to about the same value as that recorded before the anaerobic period. The inference is that in these starved leaves it was derived, as in previous periods in air and nitrogen, from turnover in the nitrogen fraction.

Direct evidence that the ethanol is oxidatively consumed to yield CO_2 in rhododendron leaves is slight. Clarke (6) could find little evidence of the oxidative consumption of ethanol from gas exchange measurements when he supplied it, in air, to discs cut from the leaves. However, when he supplied ethanol-1-C^{14} to the leaves in air some C^{14} was incorporated into acids of the tricarboxylic acid cycle (and into some unidentified components) implying that the leaves possessed at least a slight capacity for oxidation of ethanol in reactions of the cycle. Similar experiments using ethanol-C^{14} with other plants (e.g. 9, 10) suggest also that ethanol is oxidatively consumed by way of the tricarboxylic acid cycle. In the present experiments the RQ values after the first hour in air rapidly returned to values (0.8 upwards) similar to those recorded in air before anaerobiosis. The rapid oxidative consumption of ethanol would

have been expected to depress these values at least for a period. Clearly experiments in which labeled ethanol is supplied to leaves in air immediately after a prolonged period of anaerobiosis are now indicated.

Turning to events in the first hour or so after return to air, it has been postulated that there is a third component contributing to CO_2 output. In the acid analyses there were indications for this period of the depletion of succinate and of malate and of a temporary increase in tricarboxylic acids. These changes in acid levels are consistent with a restarting of carbon traffic through the tricarboxylic acid cycle when O_2 once again became available to sustain the oxidative sequences. The O_2 uptake rose to a maximum more quickly than the CO_2 output on return to air; consequently, the initial RQ values were near zero, but within an hour or so rose to values between 0.8 and 1.0. If the tricarboxylic acids accumulated at the expense of dicarboxylic acids, e.g. succinate and malate, and acetyl units derived from carbohydrate, the RQ of the combined reactions would lie between 0.6 and 0.7. If however, the acetyl units were derived from ethanol, the RQ would be zero. A combination of such reactions would conceivably account for the rapid O_2 uptake on first return to air and for the initial low RQ values. The major part of the CO_2 output consequent upon these reactions would come later when the tricarboxylic acids were decarboxylated in further reactions of the cycle.

Against this explanation, however, are the changes in concentration observed for the keto-acids. These may well be a better guide to changes of flux through the cycle than are changes in levels of the other acids, which may be present largely in inactive pools. There were no spectacular changes in the levels of any of the keto-acids to indicate a rapid resurgence of cycle activity on return to air. Barker and Younis (3) in their experiments using inhibitors on strawberry leaves found some correlation between the rate of CO_2 output and the concentration of oxaloacetate and pointed out that for the strawberry leaf the level of oxaloacetate appeared to provide an index of the rate of traffic through the cycle. In the rhododendron experiments the oxaloacetate increased only slowly from a low level to the air-line value in the course of 24 hours.

The experiments in which labeled glucose was supplied to the leaves after return to air from nitrogen, showed very little utilization in the first few hours of the sugar supplied, and as noted earlier, there was no evidence from analysis of reserves of carbohydrates present in the leaves of carbohydrate utilization in this period. Each of the glycolytic intermediates measured, with the exception of fructose-1,6-diP, increased transiently on return to air suggesting possibly some initial retardation of glycolysis.

One thing is clear however and that is that the re-establishment of new steady state concentrations of the intermediates, to be expected if glycolysis settled rapidly to some new steady rate, was not approached quickly on return to air.

There are clearly many unsolved problems remaining for these leaves. A major part of the CO_2 output at the various stages of these experiments appears to have been derived from constituents not investigated so far. Further analyses are now required, particularly for additional reserves of carbohydrate nature and for nitrogenous reserves.

Summary

Detached leaves of *Rhododendron ponticum* evolved CO_2 in air for long periods, the RQ falling to, then remaining at, about 0.8. In nitrogen, or oxygen concentrations less than 1.6 %, the CO_2 output was enhanced initially and ethanol accumulated. The molar quotient, ethanol/CO_2, in the absence of oxygen was about 0.6 and remained less than unity for periods in excess of 24 hours. Acetaldehyde and lactate were present in trace amounts only. On return to air from nitrogen the CO_2 output was enhanced and the ethanol disappeared slowly over periods of days. The RQ was initially very low but rose within about 1 hour to values of 0.8 to 1.0.

Carbohydrate consumption was markedly increased on transfer from air to nitrogen, indicating the operation of a Pasteur effect. Measured carbohydrate consumption accounted for only part of the respiratory carbon loss in air and nitrogen; at times for none of it in these gases and for none of it on first return to air from nitrogen. Observed changes in total tricarboxylic cycle acids were slight throughout.

There were rapid adjustments in levels of glycolytic intermediates on transfer from air to nitrogen and on return to air suggesting marked adjustment rates of glycolysis. Rapid changes in 6-P-gluconate together with different $C^{14}O_2$ yields from glucose-1-C^{14} and -6-C^{14} indicate varying contributions of the pentose phosphate cycle.

Transient changes in acid levels on return to air were consistent with oxidations of the tricarboxylic acid cycle and with the initial low RQ's. Slow adjustments of the levels of keto-acids on return to air, however, suggest slow resumption of cycle activity. Very little of the labeled glucose supplied at this stage was consumed.

A major problem remaining is the nature of the endogenous respiratory substrate.

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