THE RESPIRATION OF *ARUM* SPADIX. A RAPID RESPIRATION, RESISTANT TO CYANIDE

By W. O. JAMES and H. BEEVERS

Oxford Medicinal Plants Scheme, Department of Botany, University of Oxford

(*Received* 13 *March* 1950)

(With 16 figures in the text)

It has long been known that the spadix of *Arum* has an unusually fast rate of respiration while the inflorescence is developing. Church (1908) speculated that the resulting rise of temperature within the opening spathe, amounting almost to 20° C. in *A. italicum*, might have biological significance in attracting pollinating insects to the flowers; but the metabolic mechanism of the respiration has not previously been studied. Van Herk (1937) has made a notable investigation of the corresponding tissue in the tropical aroid *Sauromatum*, and his results will be discussed after describing our own.

Material

The sterile region of the *Arum* spadix has been examined in varying stages of development, as shown in Fig. 1α–ζ. The material was mostly growing wild, and was collected from typical sites around Oxford. It was therefore not possible to assess accurately the average time interval between the stages selected, and the intervals may not have been equal between each pair of stages. Nevertheless, by assuming them to be equal, one may plot a developmental curve, such as Fig. 2, on a morphological scale which can depart in no significant respect from a corresponding curve drawn to a simple time scale.

The respiration was examined by means of slices of tissue about 40 μ thick cut freehand across the mid-region of the spadix, by means of crude saps, and by various types of partially purified extracts. Comparative experiments were carried out with adjacent tissues, viz. the enveloping spathe and the stalk on which the inflorescence is supported.

Most of the experiments were performed with *A. maculatum* L. or *A. italicum* Mill. Other species examined briefly for comparison were *A. creticum* Boiss. & Held, *Arisaema amurense* Maxim., *Biarum tenuifolium* Schott, *Amorphophallus rivieri* Dur. and *Sauromatum* sp.

Methods

Oxygen consumption was measured in Warburg manometers at 30° C. All flasks were equilibrated by shaking for at least 15 min. in contact with air or the gas mixture to be employed. Tissue slices, washed in three changes of distilled water, and extract were suspended in M/15-phosphate buffer at pH 6, except where otherwise stated.

Carbon dioxide production was measured under similar conditions by the two-flask method. Saps and other extracts could be accurately pipetted to give equal aliquots. Tissue experiments were done with samples of fifteen slices selected at random from a large number previously prepared and washed. The sampling error involved by this technique was tested with five samples of disks cut from an αβ spadix and washed in the

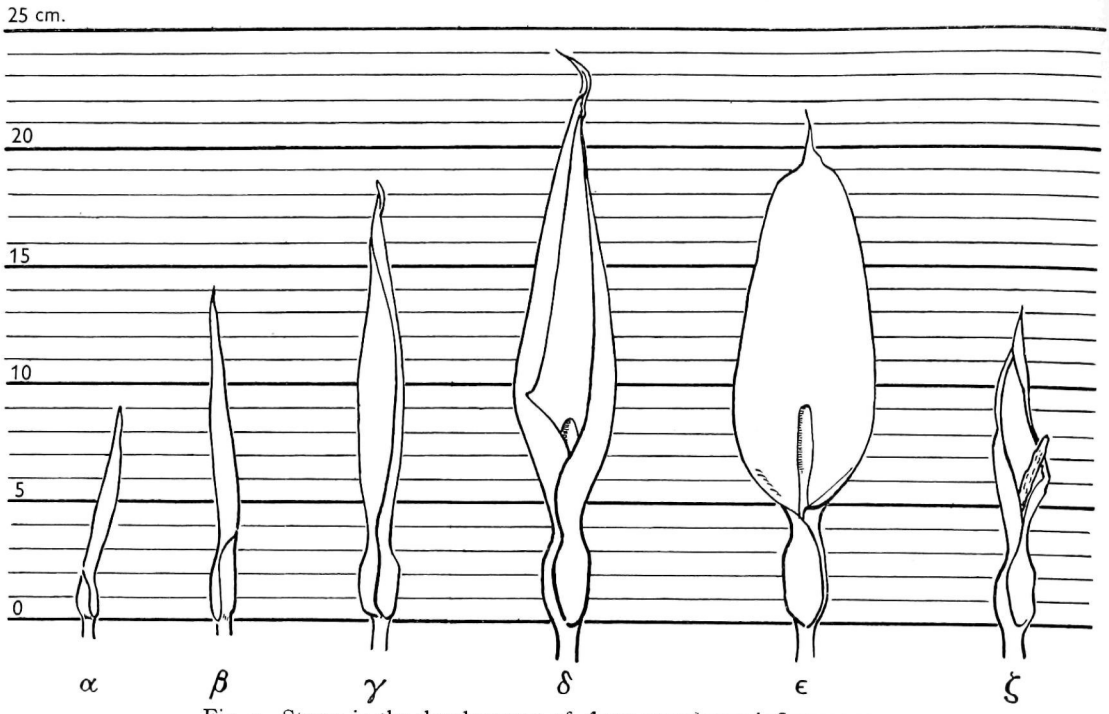

Fig. 1. Stages in the development of *Arum maculatum* inflorescences.

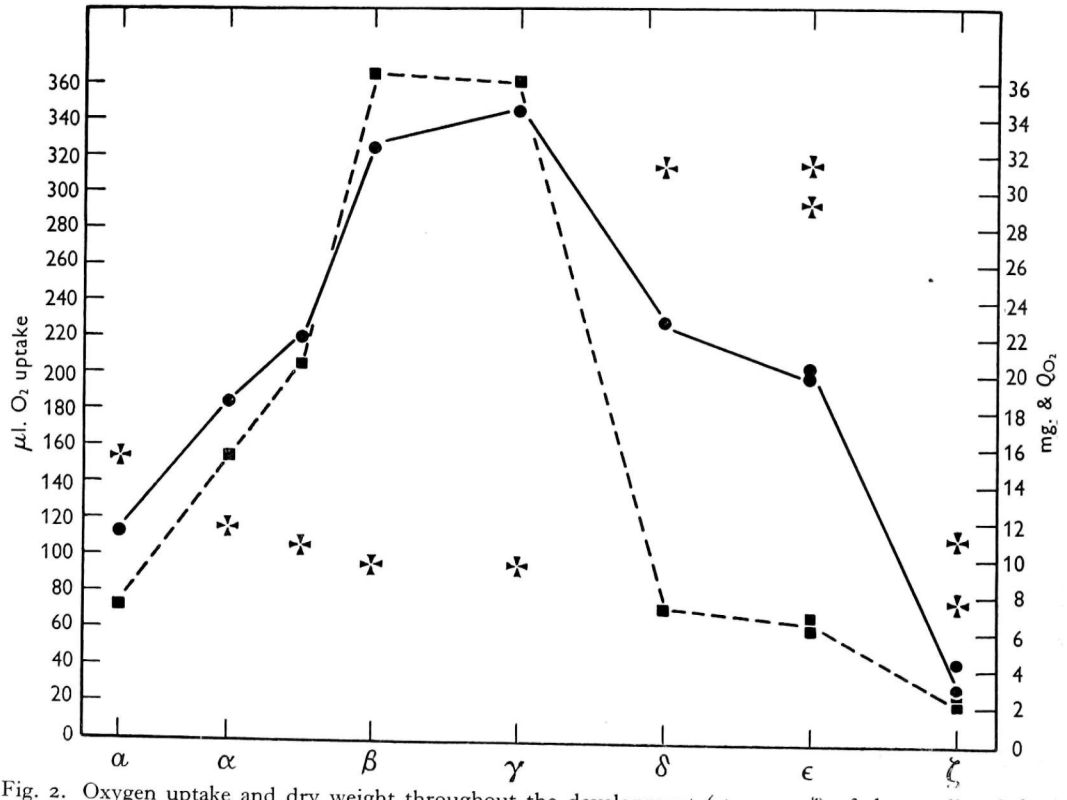

Fig. 2. Oxygen uptake and dry weight throughout the development (stages α–ζ) of the spadix of *Arum maculatum*. ●—● μl. oxygen/hr./15 slices; ■ – – ■ dry weight of 15 slices in mg. (right-hand scale); ✠ ✠ Q_{O_2} (right-hand scale).

usual manner. Each sample was placed in a separate manometer vessel, and the oxygen uptake measured under the usual conditions at 5 min. intervals for three-quarters of an hour. The slopes of the best straight lines through each set of nine points were obtained and gave the following rates of oxygen consumption per sample: 3·66, 3·63, 3·70, 3·61, 3·56 μl. O_2/min.; mean = 3·63 and standard deviation 0·06, less than 2% of the mean. In general, deviations exceeding 5% from a control would therefore have a high degree of significance, and errors in carbon dioxide estimations due to differences between the paired samples would be negligible.

Application of cyanide. As caustic alkali was present in the centre cups of the manometric flasks to act as carbon dioxide absorbent, precautions had to be taken to prevent distillation of cyanide out of the medium in which the tissue slices were immersed. Krebs (1935) and Umbreit, Burris & Stauffer (1945) have proposed to do this by adding equilibrium amounts of potassium cyanide to the alkali beforehand. Recently, some features of these methods have been criticized (Laties, 1949). We preferred to give our tissue slices an initial period of 2 hr. immersion in the medium containing a known concentration of cyanide before the potassium hydroxide was put into the centre cup. A further 15 min. equilibrium time was allowed, and then readings of oxygen uptake were taken over a short period. The oxygen uptake of yeast submitted to this procedure showed strong inhibition and no tendency to recover during the next 30 min. The method appears, therefore, to give a reasonably good estimate of the degree of inhibition produced by a stated concentration of cyanide. Other inhibitors were added in routine fashion, except that some of the experiments were carried out under more acid conditions. Turner & Hanly (1947) obtained their best malonate inhibition of carrot respiration in acid solutions, and it has been shown that other weak acid inhibitors are most active at pH values where they exist largely in the undissociated form (Beevers & Simon, 1949). The experiments with malonate and fluoride inhibitors were therefore carried out at pH 3·5.

Results

Experiments with tissue slices

Oxygen consumption. Samples of spadix slices of *Arum maculatum* or *A. italicum*, prepared and washed as above, were suspended in M/15-phosphate buffer in manometric flasks, and oxygen uptake was measured in the usual way. The rates of uptake were rapid, and were almost constant over periods of 5–6 hr. (cf. Figs. 8, 9). The rate of uptake by fifteen slices of an $\beta\gamma$ spadix was 355 μl./hr. at pH 7·4 and 328 at pH 3·5. The rate is therefore high over a wide range of acidities.

Stage of development of the spadix. The oxygen consumption, carbon dioxide output, and respiratory quotients (R.Q.) of fifteen tissue slices were measured at eight successive stages of development of the spadix. The stages labelled α–ζ are illustrated in Fig. 1. A very early stage, not drawn, has been labelled *a*, and a stage that appeared to be intermediate between α and β has been labelled $\alpha\beta$. These results are recorded in Table 1 together with the dry weight of the tissue samples and the calculated rates of oxygen consumption per mg. dry weight per hour (Q_{O_2}) and shown graphically in Fig. 2.

It will be seen that the rate of respiration per slice increases rapidly while the inflorescence is enlarging (stages α–γ). At the period when the spathe begins to open and the spadix has reached its full size (stage δ), respiration per slice begins to slow down. The dry weight of the slices follows the same sequence, the fluctuation being mainly due to an initial

accumulation of starch and its subsequent disappearance. The Q_{O_2} is strikingly influenced by this factor and remains approximately constant up to stage γ; it then exhibits a sudden rise owing to the rapid loss of solid reserves from the tissues.

A clearer picture is presented by the curve for oxygen consumption per slice, which shows that the respiration rate has already climbed to a peak before the sudden rise of Q_{O_2} occurred.

Substrate of respiration. During the washing of slices from spadices at stages β and γ, enough starch escaped from the cut cells to make the washings thickly turbid and, on staining with iodine, the intact cells were seen to be filled with fine starch grains. At later stages the washings remained almost clear, since the cells were no longer packed with the grains. There was at the same time a heavy loss of dry weight, and the R.Q. dropped for the first time below 1·0. It seems clear that the rapid respiration was maintained at the expense of the starch, and that lack of carbohydrate was the primary cause of its falling off in the

Table 1. *Respiration of spadix slices of* Arum maculatum

Stage	Dry weight 15 slices (mg.)	Oxygen uptake (μl. O_2/15 slices/hr.)	Carbon dioxide output (μl. CO_2/15 slices/hr.)	Q_{O_2}	R.Q.
a	7·2	112	—	15·6	—
α	15·7	183	—	11·7	—
$\alpha\beta$	20·4	218	—	10·7	—
β	36·4	326	355	9·8	1·09
γ	36·0	344	348	9·6	1·01
δ	7·2	228	219	31·6	0·96
ϵ	6·4	203	—	31·8	—
ϵ	6·8	200	178	29·4	0·89
ζ	3·4	45	—	13·2	—
ζ	3·8	29	25	7·8	0·87

later stages. This was confirmed by adding 0·2% glucose to the external medium. At stages earlier than ϵ this produced no increase in the rate of oxygen uptake; at stage ϵ 23% increase and at stage ζ 130% increase.

Oxygen concentration. Tissue slices were suspended in a buffer in manometric flasks with caustic potash in the centre chambers. The air in the flasks was replaced with oxygen or a previously prepared mixture with air or commercial nitrogen, and the flasks were then brought to equilibrium by 15 min. shaking. The manometers were then levelled and closed, and readings were begun. Oxygen uptake by *A. maculatum* slices was linear with time (Fig. 3) in concentrations from 10·5 to 100% oxygen (1 atm. pressure). When slices were returned to air from other gas mixtures they immediately resumed the normal rate of oxygen uptake in air.

The slopes in Fig. 3 correspond to the following rates of oxygen uptake:

10·5	21	100% oxygen
146	300	980 μl. O_2/15 slices/hr.
10	21	67 relative rates

There is a doubling of the rate between 10·5 and 21% O_2, and a further large increase in rising to 100% O_2. It will be seen that measurements of Q_{O_2} of tissues with a high air value (e.g. $Q_{O_2} > 30$, as δ in Table 1) would give values of as much as 100 in 100% O_2. The results of further experiments using eight different oxygen concentrations are plotted in Fig. 4, curve (*a*). It is clear that oxygen concentration exercises a marked effect on the

respiration rate at all concentrations. The large increases of oxygen uptake were accompanied by corresponding outputs of carbon dioxide, and even at 100% oxygen there was no significant fall in the R.Q. (Table 2).

This extreme dependence on oxygen concentration is a peculiarity of the spadix respiration, and was not shown by that of adjacent tissues. A similar series of experiments

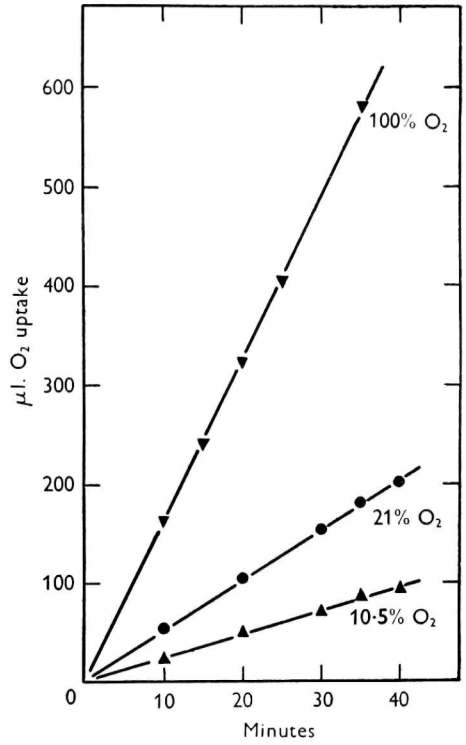

Fig. 3. Effect of variation of oxygen concentration on oxygen uptake of slices of spadix of *Arum maculatum*.

Table 2. *Effect of oxygen concentration on respiration of* Arum *spadix slices* (*stage* β–γ)

Oxygen %	Oxygen uptake (μl. O_2/15 slices/hr.)		R.Q.
2·0	48	—	—
4·0	66	—	—
10·5	156	—	—
21·0	306	354	1·11
35·0	—	456	—
50·0	—	630	1·00
66·0	—	730	—
100·0	—	970	0·97

was performed using slices of the stalk supporting the inflorescence. The rates of oxygen uptake observed are plotted in Fig. 4, curve (*b*). The rates per slice and the Q_{O_2} values are much lower than those for spadix slices (Table 3). The two curves have therefore been plotted to coincide at the air value (made equal to 100) in order to display more clearly their different forms. At 21% oxygen the respiratory system of the peduncle is already saturated, whereas that of the spadix is not saturated at 100% (1 atm. pressure). The design of the experiments precludes the possibility of these differences depending on diffusion; they must be ascribed to different oxidation mechanisms.

Anaerobic respiration. Simultaneous experiments were carried out with spadix slices of *A. italicum* under air and nitrogen. Duplicate flasks were set up with air, and another pair with nitrogen. After 30 min. measured respiration, the air in one flask was replaced

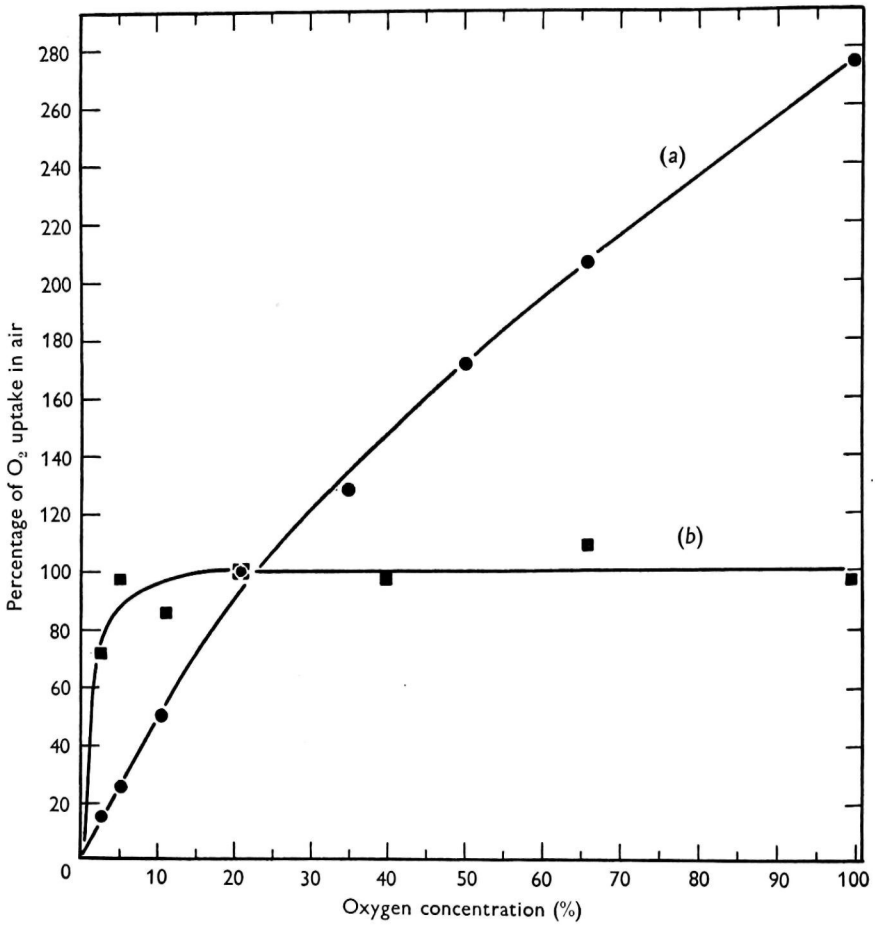

Fig. 4. The effect of oxygen concentration on uptake of oxygen by (*a*) spadix slices and (*b*) stalk slices. The curves are plotted to coincide at the air value. For actual values see Tables 2 and 3.

Table 3. *Effect of oxygen concentration on respiration of* Arum *peduncle slices*

Oxygen (%)	Oxygen uptake (μl. O_2/25 slices/hr.)	Q_{O_2}
2·5	29	—
5·0	37	—
10·5	32	—
21·0	37	2·94
40·0	36	—
66·0	41	—
100·0	37	(2·94)

with nitrogen, and one of the nitrogen flasks was aerated. Respiration readings were resumed after 20 min. The other two flasks were maintained with their initial atmosphere throughout. Carbon dioxide output was measured directly under nitrogen, and by difference between the two flasks in air, one having caustic potash in the centre chamber

and the other not. Oxygen uptake in air was measured directly in the flasks containing caustic potash. The results are given in Table 4. The carbon dioxide output in air remained approximately linear, and even increased slightly with time; under nitrogen there was a much slower output. The values for the ratio CO_2 output in nitrogen/CO_2 output in air (I/N) at succeeding time intervals show fluctuations in the range 0·3–0·4. These values are close to the theoretical value 0·33 corresponding with a simple transition to alcohol formation under nitrogen without further complications, such as change of glycolysis rate. Measurements of alcohol formation have not yet been carried out, so the suggestion remains unconfirmed.

The effect of cyanide. Cyanide was applied to spadix slices of *A. italicum* at stage β–γ by the method detailed on p. 355. The usual 'differentiating' concentration of M/1000 was employed and also M/500 and M/100. The rates of oxygen absorption by fifteen slices are shown in Fig. 5A. The higher concentrations were used because some polyphenolases have been found to be somewhat resistant to cyanide poisoning, and their absence cannot

Table 4. *Respiration of spadix slices of* Arum italicum *in air and nitrogen*

Flask	Atmosphere	KOH	Measurement	Gas exchange of 15 slices				
				10	15	20	25	30 min.
I	Air	+	O_2 uptake	62	98	131	164	204 μl.
II–I	Air	o	CO_2 output	70	111	148	185	231 μl.
III	Nitrogen	o	CO_2 output	29	37	45	57	66 μl.
IV	Nitrogen	o	CO_2 output	27	50	62	75	85 μl.
III/II	—	—	I/N	0·41	0·33	0·30	0·31	0·29
IV/II	—	—	I/N	0·39	0·45	0·42	0·41	0·37

safely be inferred from lack of inhibition at M/1000. There was no inhibition at M/1000 or M/500 and only very slight inhibition (14%) at the high concentration. The effect of cyanide on the respiration of *A. maculatum* slices was examined at different stages of development of the spadix. The results, listed in Table 5, show that at M/1000 the effect was either trifling or nil. Even with M/200 cyanide, no inhibition could be demonstrated at stages β and γ, though there appeared to be some inhibition in the earliest and latest stages with this high concentration.

The effect of cyanide on spadix slices of other aroids was also examined with the results listed in Table 6. No large inhibitions were observed.

The effect of diethyldithiocarbamate. Since no inhibitions were observed with cyanide, none would be expected with this more specific copper inhibitor. Several experiments were carried out in the usual manner with carbamate present throughout the period of measurement. No measurable depression of the oxygen uptake was observed with M/1000 or M/500 diethyldithiocarbamate applied to *Biarum tenuifolium* slices, and with *Aerissima amurense* the reductions were 8% at M/500 and 10% at M/200.

Effect of carbon monoxide. Manometric vessels containing samples of fifteen slices of spadix were filled and equilibrated with the following gas mixtures: (*a*) 80% CO + 20% O_2; (*b*) 96% N_2 + 4% O_2; (*c*) 80% CO + 4% O_2 + 16% N_2. A control sample, (*d*), was run in air. The rates of oxygen uptake observed are recorded in Fig. 6, and it will be seen that neither at 20% O_2 nor at 4% O_2 was there any reduction due to carbon monoxide.

The effect of malonate and succinate. Fig. 7 shows the results of an experiment with three flasks containing slices suspended at pH 3·5 (see p. 355). To one M/20-malonate was

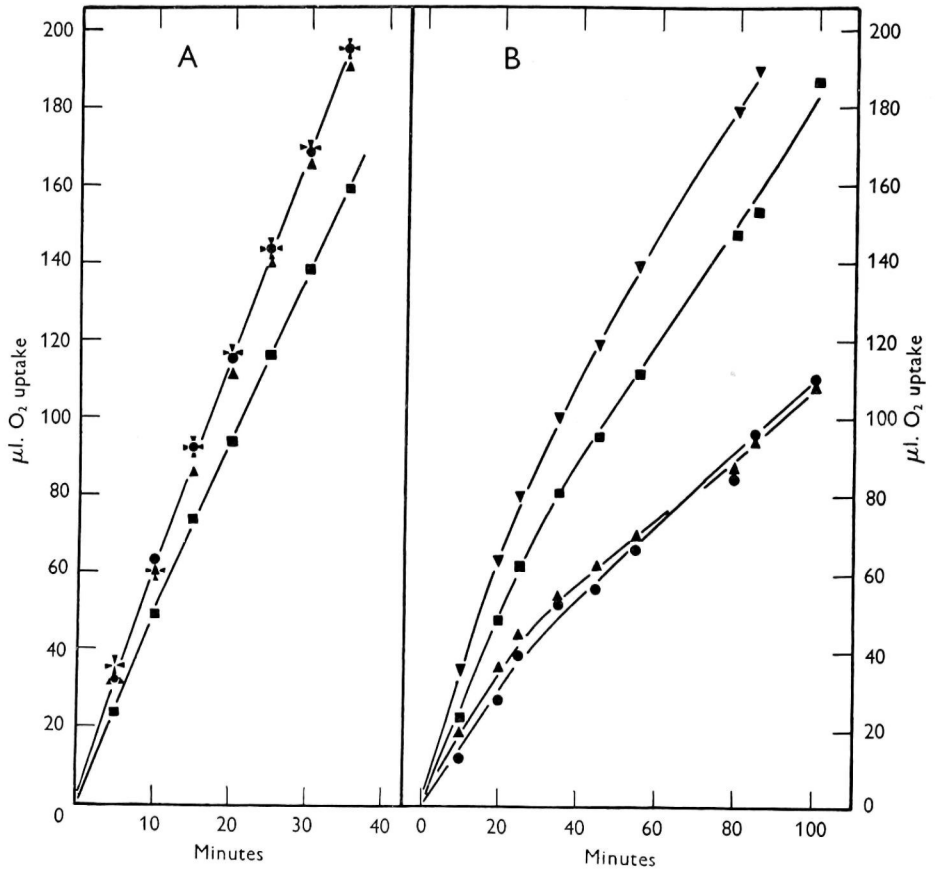

Fig. 5. A. Effect of cyanide on oxygen uptake by slices of spadix of *Arum italicum*. ●—● No cyanide; ▲—▲ M/1000-cyanide; ✠—✠ M/500-cyanide; ■—■ M/100-cyanide. B. Effect of cyanide and hexosediphosphate on oxygen uptake of extract from spadix of *A. italicum*. ●—● 2 ml. extract only; ▲—▲ extract + M/500-cyanide; ■—■ 2 ml. extract + approx. 4 mg. hexosediphosphate; ▼—▼ 2 ml. extract + 4 mg. HdP + M/500-cyanide.

Table 5. *Percentage inhibition of oxygen uptake by spadix slices of* Arum maculatum *by* M/1000-*cyanide*

Stage of development (Fig. 1)	*a*	$\alpha\beta$	γ	ϵ	ζ
Percentage inhibition	10	11	nil	nil	nil

Table 6. *Inhibition of oxygen uptake by fifteen spadix slices of various aroids by* M/500 *and* M/200-*cyanide*

Species	Oxygen uptake μl./hr.			Percentage inhibition	
	No cyanide	M/500-cyanide	M/200-cyanide	M/500	M/200
Aerissima amurense	78	73	72	6	8
	240	244	—	nil	—
Amorphophallus rivieri	*221	—	274	—	nil
Arum creticum	*180	—	173	—	4
Biarum tenuifolium	64	—	61	—	5
	*130	—	117	—	10

* Glucose added to the medium to a concentration of M/10.

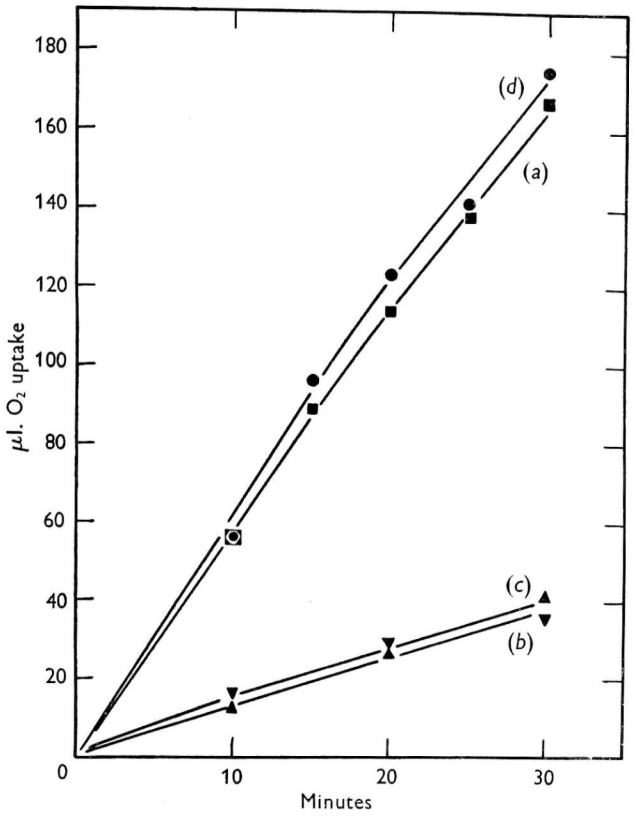

Fig. 6. Effect of carbon monoxide on oxygen uptake of slices of spadix of *Arum maculatum*. (*a*) 80 % carbon monoxide + 20 % oxygen; (*b*) 96 % nitrogen + 4 % oxygen; (*c*) 80 % carbon monoxide + 4 % oxygen + 16 % nitrogen; (*d*) air.

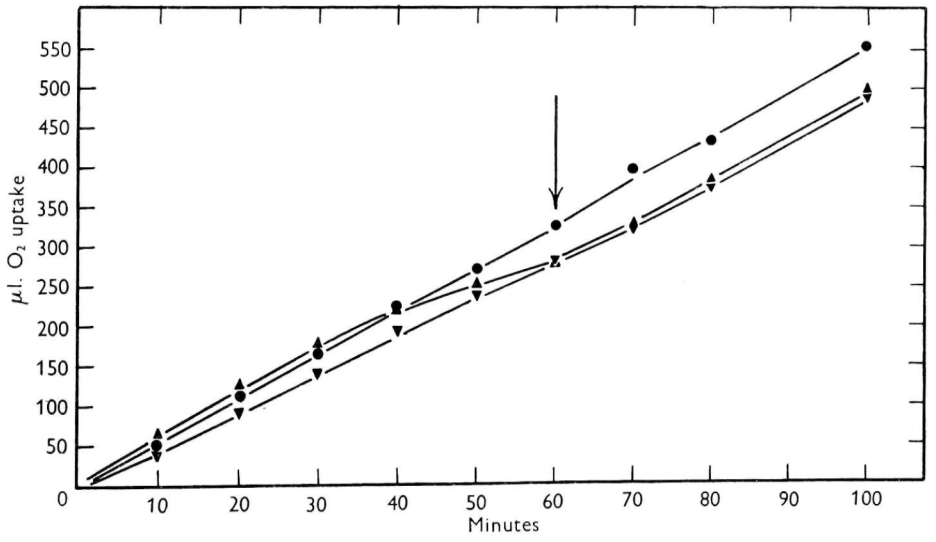

Fig. 7. Effect of malonate and succinate on oxygen uptake of slices of spadix of *Arum italicum*. ●—● No malonate, M/100-succinate added after 60 min. as shown by arrow; ▲—▲ M/100-succinate present throughout, M/20-malonate added after 60 min.; ▼—▼ no succinate, M/20-malonate throughout.

added, to the second M/100-succinate and to the third neither. The control and succinate flasks showed almost identical rates of oxygen uptake and the malonate flask a steady rate only 14% below the control. After 60 min. succinate was added to the control flask and malonate to the succinate flask. Neither of these additions produced deflexions of the curves. It therefore seems clear that the respiration of the spadix slices is neither inhibited by malonate nor accelerated by succinate.

The effect of iodoacetate. Experiments were performed in the usual way with M/5000, M/1000 or M/500-iodoacetate in the side arms of the flasks. Tissue slices of both *Arum italicum* and *A. maculatum* were examined and the results are shown in Fig. 8A and B.

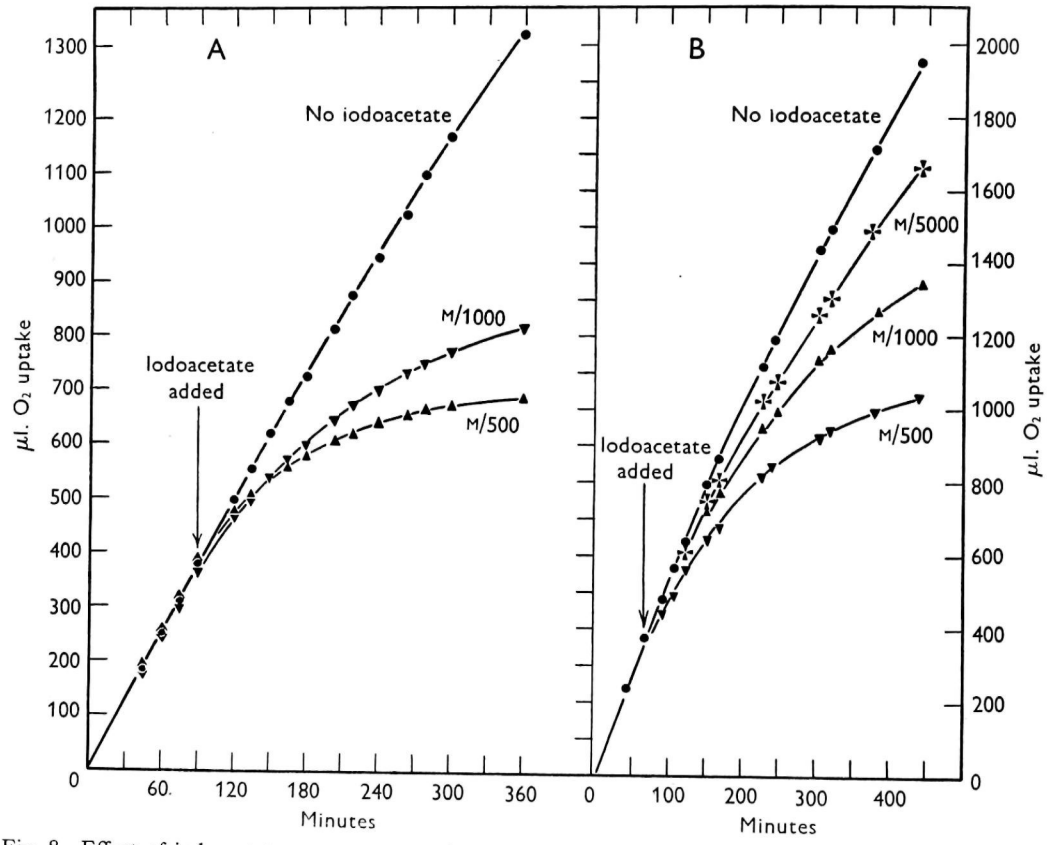

Fig. 8. Effect of iodoacetate on oxygen uptake of slices of spadix. A, *Arum italicum*. B, *A. maculatum*. Iodoacetate added as shown by arrows.

Iodoacetate is known to be slow in entering plant tissues (cf. Turner, 1937), and the experiments were therefore carried on for longer than usual. With all flasks the uninhibited respiration rate was first established, and iodoacetate was then tipped into the medium from the side arm. Reduction of the rate slowly developed in every instance and was greatest with the highest concentration. A 50% reduction was achieved with M/1000-iodoacetate after 4 hr., and with M/500 it was almost complete. After 23 hr. the rate of oxygen uptake was constant in all the flasks, and the concentration of iodoacetate required for 50% inhibition at pH 6·0 approximated closely to 5×10^{-4}M.

Effect of fluoride. Preliminary experiments showed that the oxygen uptake of tissue slices was strongly inhibited by M/50-fluoride at pH 3·5. In a more detailed experiment slices

of *A. italicum* spadix were allowed to respire for 45 min. in air. A control sample was continued for 285 min. and, in two other flasks, the inhibitor was tipped in from the side arm. In one the concentration of fluoride was M/250 and in the other M/500. The time courses of the oxygen uptakes are shown in Fig. 9A. At the end of the period, inhibition

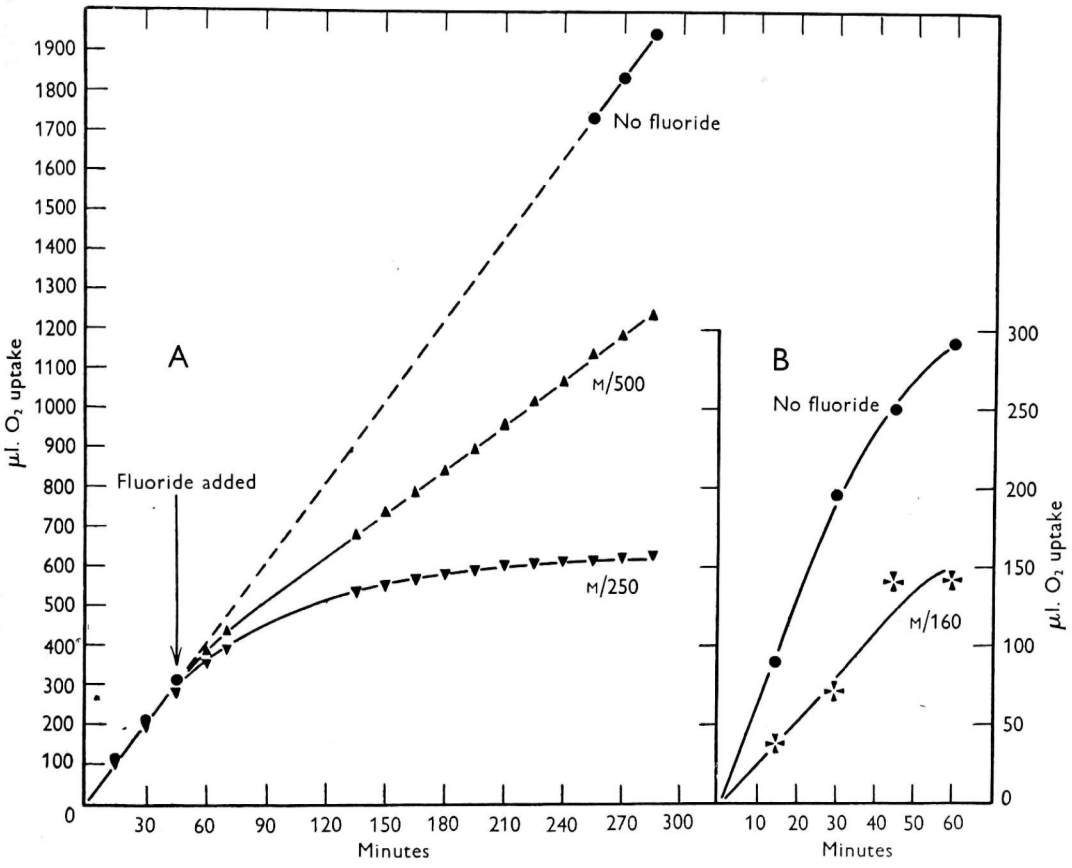

Fig. 9. Effect of fluoride on oxygen uptake. A, spadix slices of *Arum italicum*, fluoride added as shown by arrow. B, extract from spadix of *A. italicum*, fluoride present from start.

Table 7. R.Q. *of slices of* Arum italicum *spadix in the presence of sodium fluoride at* pH 3·5. *Oxygen uptake and carbon dioxide output in µl. Sodium fluoride added after* 45 *min.*

Period (min.)	0–45			60–135			135–180			180–225		
	O_2	CO_2	R.Q.	O_2	CO_2	R.Q.	O_2	CO_2	R.Q.	O_2	CO_2	R.Q.
Control	300	332	1·11	500	577	1·15	315	360	1·14	315	362	1·15
M/500-NaF	300	326	1·09	290	278	0·91	167	151	0·90	172	161	0·93
M/250-NaF	300	326	1·09	181	180	1·00	49	43	0·88	28	23	0·82

due to M/250-fluoride was 90% and that due to M/500 was 45%. The R.Q.'s were also determined in the same series, with the results shown in Table 7.

As inhibition develops a definite fall of the R.Q. becomes apparent because the output of carbon dioxide falls off more than the consumption of oxygen. Since fluoride is supposed to inhibit enolase (Warburg & Christian, 1942), this is intelligible on the likely

assumption that some oxygen is consumed and no carbon dioxide is released before pyruvic acid is formed.

Effect of oxidase inhibitors on other tissues. The rates of oxygen uptake per slice and the Q_{O_2} values were much lower in surrounding tissues than in the spadix itself, and were markedly reduced by cyanide and diethyldithiocarbamate. The oxygen uptake of slices of the flower stalk of *A. italicum* was inhibited 48% by M/1000-cyanide. Similar slices from *A. maculatum* with a $Q_{O_2}=2{\cdot}7$ showed an inhibition of 64% in the presence of M/250-diethyldithiocarbamate. The oxygen uptake of slices from the petioles of winter leaves of *A. italicum*, in a medium containing glucose, was inhibited 67% (from 73 to 24 μl./hr./25 slices) by M/250-cyanide. These results contrast strongly with those of Tables 5 and 6 for spadix slices, and are more in agreement with results from other plant tissues.

EXPERIMENTS WITH CELL-FREE EXTRACTS

Extracts were prepared from spadices of $\beta\gamma$ stage in two ways: (*a*) Young spadix tissue was frozen at $-6°$ C. overnight and allowed to thaw at room temperature. It was then ground in a mortar, and the extract strained through muslin and centrifuged for a few minutes at 2000 r.p.m. (*b*) Fresh tissue was ground in a mortar with enough water or phosphate buffer at pH 6 to give it a creamy consistency. It was then passed through muslin and centrifuged, as in (*a*). Copious quantities of starch came down during the centrifuging. Both methods yielded a cloudy yellow extract; on standing or on further centrifuging, a yellow flocculum settled out and its removal caused no loss of activity. The activities of successive preparations varied considerably, but the best were obtained without freezing.

These cell-free extracts continued both to absorb oxygen and to give off carbon dioxide. When a carbon dioxide absorbent was present in the flasks, oxygen uptake was observed to be more or less linear (Fig. 10, curve (*a*)). The release of carbon dioxide sometimes exceeded the uptake of oxygen so far that the R.Q. rose to 1·5; but in many preparations the gas exchange continued for several hours with almost equal carbon dioxide output and oxygen intake. Boiled extracts were found to be completely inactive.

Oxygen concentration. Aliquots of an extract, prepared from *A. italicum* spadices by method (*b*), were placed in three monometers with carbon dioxide absorbent in the centre chambers. The gas mixtures in the chambers were nitrogen, with 10, 21 and 100% oxygen respectively. The rates of oxygen uptake are shown in Fig. 10, curves (*c*), (*a*) and (*b*). The initial rates were in the ratio of 10:20:53, but there was a fairly rapid falling off, especially in 100% oxygen. These results are very similar to those obtained with slices of spadix tissue (p. 356 and Fig. 3); and it is clear that oxygen tension exercises a controlling effect even at high concentrations. Carbon dioxide output was also increased in 100% oxygen, and the gas quotient (CO_2/O_2) fell only silghtly from 0·89 in air to 0·82 in oxygen.

The effect of cyanide. In this experiment, cyanide was added directly to the extract to give a concentration of M/500, and caustic potash was put into the central cup immediately before equilibration of the manometers. Distillation of cyanide out of the extract could not have been a significant factor, since no inhibition was observed even at the start. The result is illustrated in Fig. 5 B. Other experiments in which cyanide was applied to extracts are described in later sections.

The effect of iodoacetate. The addition of M/1000-iodoacetate (final concentration) to the systems whose O_2 uptakes are shown in Fig. 12, immediately produced large-scale inhibitions. The activities of the systems in the 30 min. after tipping were all less than

30% of those in the 30 minutes period prior to the addition (90–120 min. in Fig. 12); with the exception of (*e*) in which the already very slow rate was diminished by only one-fifth.

The effect of fluoride. In the experiments reported with tissue slices fluoride was used at pH 3·5. The tissue respired actively in phosphate buffer of this acidity, but the extracted saps lost their activity entirely at the same pH. Fluoride added to the sap, buffered at pH 7·8 at a concentration of M/160, was found to reduce the rate of oxygen uptake by more than 50% (Fig. 9B).

Addition of hexosediphosphate (HdP). The saps prepared by either of the two methods (*a*) or (*b*) continued their spontaneous gas exchange for long periods. It must therefore be

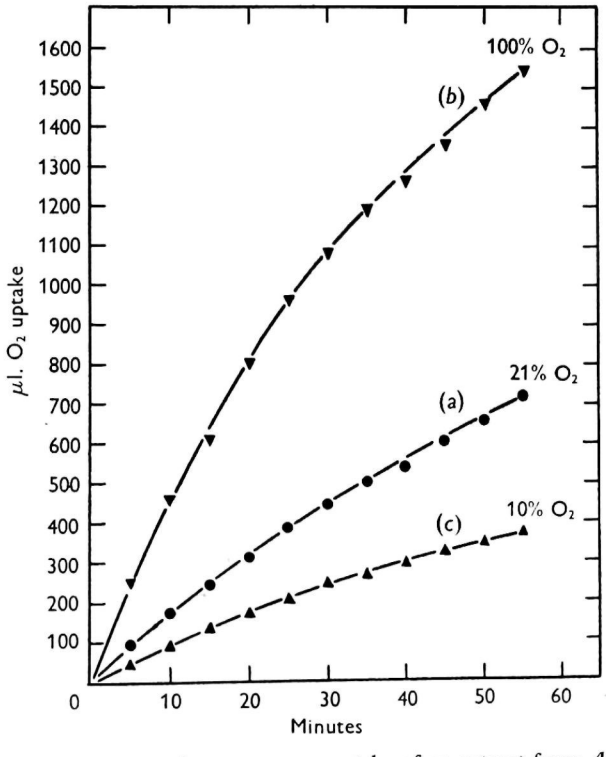

Fig. 10. Effect of oxygen concentration on oxygen uptake of an extract from *Arum italicum* spadix.

presumed that they contained supplies of appropriate substrates, as was, indeed, to be expected from the young material used. The removal of these substrates, either by chemical means or by sterile autolysis, would have been difficult, but was, in the event, found to be unnecessary.

Fig. 5B gives the results of an experiment in which hexosediphosphate was added to sap prepared by method (*a*) from *A. italicum* spadix. Barium hexosediphosphate was brought into solution in dilute HCl and the barium was removed with sulphate; 0·5 ml. of solution containing approximately 4 mg. HdP were placed in the side arm and tipped into 2 ml. extract.

It has been found in a number of experiments that a positive pressure equivalent to about 20 μl. of gas frequently develops in the first minute or so after adding hexosediphosphate to saps. In later experiments we have, therefore, left the manometer taps open

for a further period of 5 min. after adding the HdP. The cause of the initial 'back kick' is not certain; but the subsequent reduction of pressure ascribable to the hexosediphosphate addition has been greatly in excess of this. As shown in Fig. 5 B, the HdP caused a large increase of oxygen uptake. This was not inhibited by M/500-cyanide which, indeed, somewhat increased the hexosediphosphate effect. Similar stimulations of oxygen uptake on adding HdP are shown in later experiments (Figs. 11–13).

Reaction with methylene blue. When quantities of 1 or 2 ml. 0·1 % methylene blue were added to a frozen-out extract, bleaching occurred almost instantly, even when no precaution was taken to exclude oxygen. On vigorous shaking in air the colour returned, but quickly disappeared again on standing. This unusually active reduction did not survive heating, and was not due to the presence of large amounts of active non-enzymatic

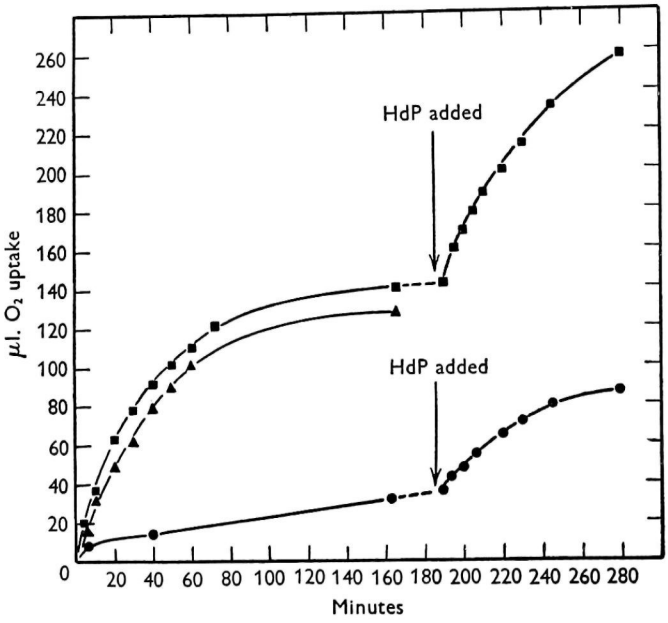

Fig. 11. Effect of methylene blue and hexosediphosphate on oxygen uptake of an extract from *Arum italicum* spadix. ●—● No methylene blue, 4 mg. HdP added after 185 min.; ▲—▲ 0·0004 % methylene blue; ■—■ 0·002 % methylene blue, 4 mg. HdP added after 185 min.

reducers. The alternate oxidation by shaking and reduction on standing could be repeated many times, until eventually the blue colour became permanent, due to exhaustion of the internal hydrogen donors.

The effect of hexosediphosphate on the decolorization of methylene blue. Methylene blue (0·5 ml. of 0·1 % solution) was added to 10 ml. of extract. Except at the exposed surface, the colour was lost almost immediately. A stream of air was then drawn through for 30 min., by which time the colour had been restored. Aliquots of the extract were then quickly pipetted into two Thunberg-Keilin tubes, one of which contained 0·5 ml. containing approximately 4 mg. HdP, while the other contained 0·5 ml. distilled water. Both tubes were exhausted and refilled with nitrogen, and, after the contents of the side arms had been tipped in, they were incubated at 30° C. The colour was completely gone from the tube containing HdP after 16 min.; but persisted in the control tube for 50 min. Hexosediphosphate was therefore able to replace the removed hydrogen donators. This was confirmed manometrically (Fig. 11).

Extract of type (*a*) was pipetted in 1·5 ml. aliquots into three manometer vessels with KOH in the centre chambers, and methylene blue was added from the side arms at the outset to give concentrations of 0, 0·002 and 0·0004% respectively. Oxygen uptake was slow in the flask containing sap alone; but initially rapid and greatly in excess of its oxygen equivalent in the presence of methylene blue. Parallel experiments disclosed that carbon dioxide output was also stimulated by such additions of dye. The rapid uptake fell away and was almost at a standstill after 160 min. At this time 0·5 ml. HdP was placed in each side arm and tipped after a further 15 min. (185 min. on Fig. 11). There was an increased

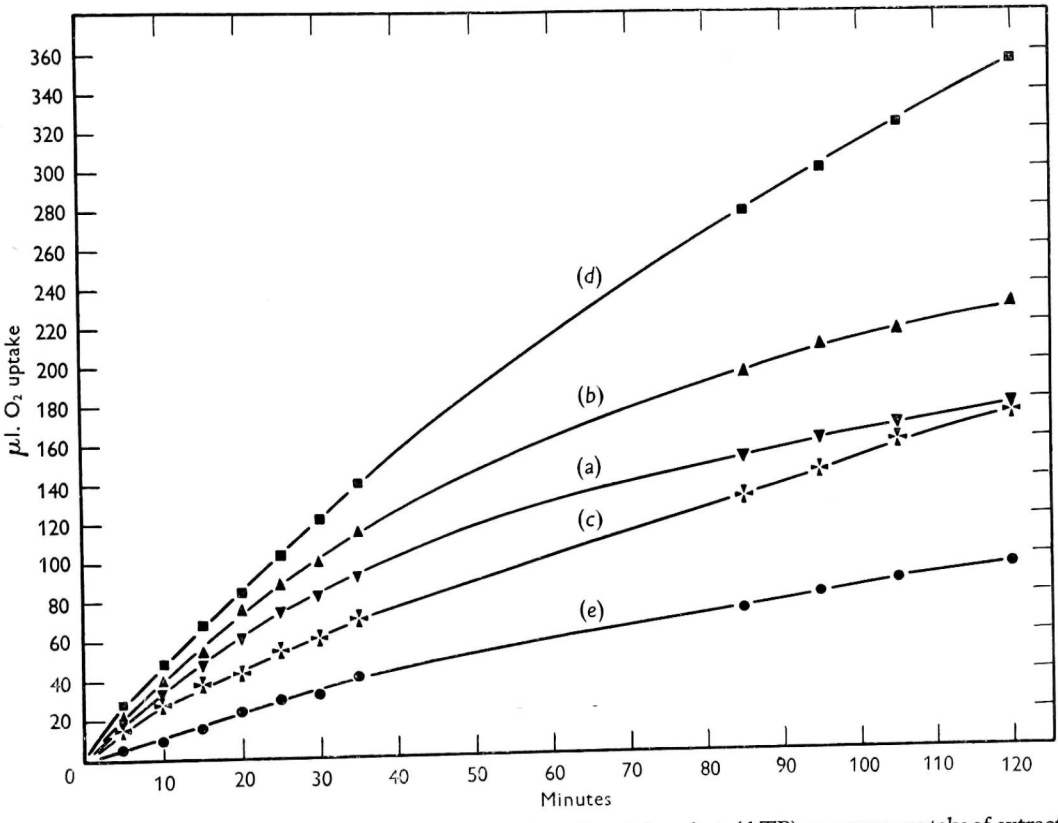

Fig. 12. Effect of hexosediphosphate, riboflavin and adenosinetriphosphate (ATP) on oxygen uptake of extract from spadix of *Arum italicum*. (*a*) 2 ml. extract + 1 mg. riboflavin; (*b*) the same + ATP (≡ 1·65 mg. barium salt); (*c*) 2 ml. extract + approx. 8 mg. HdP; (*d*) 2 ml. extract + 1 mg. riboflavin + ATP (≡ 1·65 mg. barium salt) + approx. 8 mg. HdP; (*e*) extract only.

rate of oxygen uptake in the control vessel, and an even larger one where methylene blue had been added. The ability of the HdP to act as hydrogen donor, as well as to increase the oxygen consumption of the sap, was thus confirmed. The observation that the effect of added HdP was greatest after removal of the natural substrates indicates that it competed with them for the same enzyme systems.

Addition of riboflavin and adenosine triphosphate (ATP). Aliquots of *A. italicum* extract, which had been stored at 0° C. for 3 days, were pipetted into each of four flasks. To one (*e*) there was no addition. The others received (*a*) 1 mg. riboflavin; (*b*) 1 mg. riboflavin with adenosinetriphosphate ≡ 1·65 mg. Ba salt; (*c*) hexosediphosphate ≡ 15 mg. Ba salt; (*d*) riboflavin with ATP and HdP. Over the first 30 min. the presence of the riboflavin

more than doubled the rate of oxygen uptake (Fig. 12), and in conjunction with ATP caused an almost threefold increase. The addition of HdP in (*c*) increased the rate of oxygen consumption, and the most rapid rate was shown in (*d*) which also received riboflavin. In these systems with HdP the initial rates were more nearly maintained throughout the time of the experiment, and the results show that the HdP was not acting catalytically, like the other additions, but reinforcing the consumable substrate.

In a further experiment the riboflavin was put into competition with methylene blue (Fig. 13). Freshly thawed out sap from *A. italicum* was placed in each of four vessels,

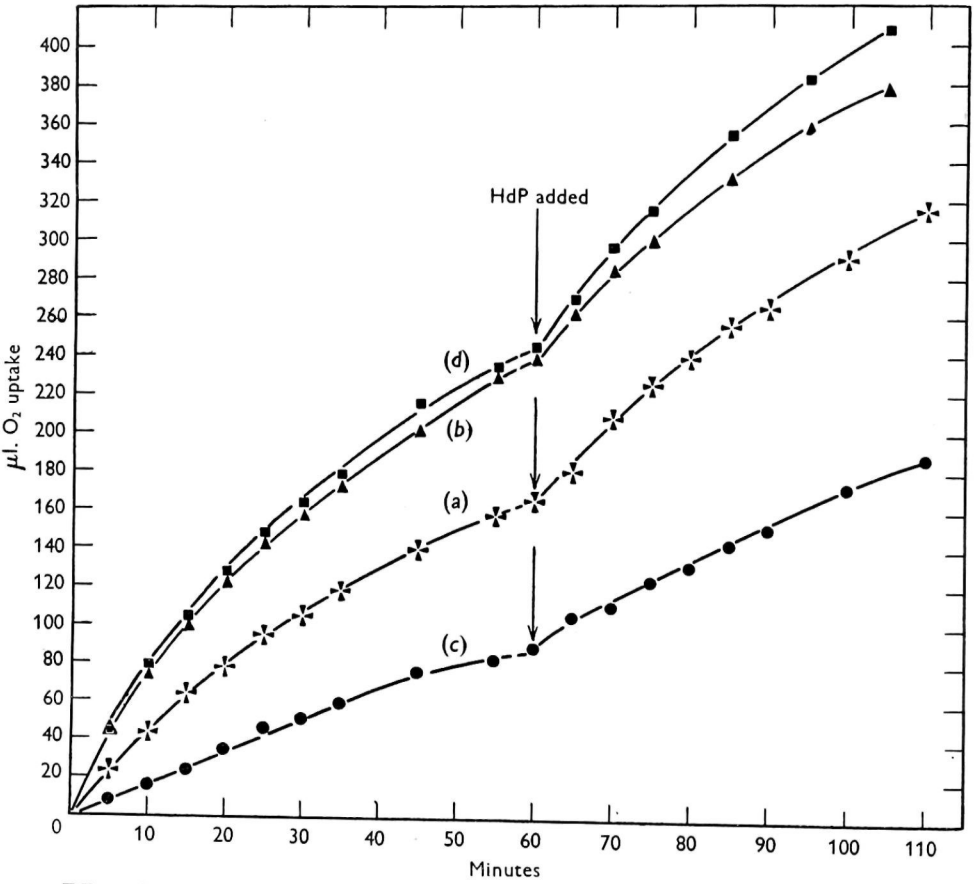

Fig. 13. Effect of methylene blue, HdP, riboflavin and ATP on oxygen uptake by an extract from *Arum italicum* spadix. (*a*) Extract + riboflavin + ATP; (*b*) extract + riboflavin, ATP and 0·002 % methylene blue; (*c*) extract only; (*d*) extract + 0·002 % methylene blue. 4·8 mg. HdP added to each after 70 min. Riboflavin and ATP as in Fig. 12.

(*a*), (*b*), (*c*) and (*d*), to two of which—(*a*) and (*b*)—were added riboflavin and ATP in the amounts described in the previous experiment. In each of the side arms of the flasks (*a*) and (*c*) was also placed 0·5 ml. containing 4·8 mg. HdP. Flasks (*b*) and (*d*) received 0·5 ml. 0·01 % methylene blue. The methylene blue was tipped at zero time and the oxygen uptake recorded. Again, the riboflavin in (*a*) almost doubled the basic control rate shown by (*c*), and there was a spectacular increase in the oxygen uptake, due to the methylene blue in (*d*). A duplicate flask without KOH showed that this uptake was accompanied by a parallel increase in CO_2 output. However, no distinction was apparent

between the rates in (*b*) and (*d*); the riboflavin did not, therefore, augment the methylene-blue effect, and the rate was the same as in its absence. After 55 min. HdP was added to the side arms of (*b*) and (*d*) and tipped into the main chamber after a further 10 min., when the side arms of (*a*) and (*c*) which contained HdP were also tipped. This addition produced a small increased in the control rate of oxygen uptake and larger increases in the presence of riboflavin, ATP and methylene blue.

Extraction of a flavoprotein. An attempt was made to isolate the oxidase responsible for the rapid oxygen uptake of the spadix. Young *A. maculatum* inflorescences were collected and spadices weighing 180 g. cut out. These were homogenized in a Waring Blendor with enough distilled water to cover them. An extract was squeezed through muslin. The residue was further extracted, with a second addition of water, by hand-grinding in a mortar, followed by a further period in the Blendor. The second extract was then squeezed through muslin as before. Both extracts were then centrifuged at 3000 r.p.m. until they were more or less clear. Basic lead acetate was then added cautiously and the precipitate removed by centrifuging. These operations were repeated until there was no further precipitation with lead. The excess lead was removed with a few drops of saturated sodium phosphate. The solution thus obtained was quite clear. It had a strong yellow colour and a pronounced green fluorescence. The colour was not bleached by ascorbic acid, but disappeared immediately on cautious additions of sodium hydrosulphite. The colour and fluorescence rapidly returned when the solution was shaken in air, and the cycle could be repeated many times. The pigment did not pass through a parchment membrane during 2 days' dialysis. The solution thus had the properties to be expected if an autoxidizable flavoprotein was present. Attempts to precipitate this with saturated ammonium sulphate or with acetone and CO_2 at O° C. (Warburg & Christian, 1932) were not successful, and the season was too far advanced to permit further efforts on a larger scale.

The absence of metallo-enzymes from spadix tissues. Preparations were made from *Arum* spadix tissue by the method of Hill & Bhagvat (1939). These were examined manometrically for oxygen uptake, with addition of *p*-phenylenediamine and cytochrome *c*. There was no oxidation of the *p*-phenylenediamine, either with or without the cytochrome (Fig. 14), and the absence of cytochrome oxidase is to be inferred. Furthermore, extracts of all types from spadix tissue have repeatedly failed to produce any colour in the dimethylamine test for catechol oxidase (Beevers & James, 1948).

A sap extraction was made from a spadix of *Sauromatum* sp. It showed no ability to oxidize catechol. There was a slow oxidation of ascorbic acid, but no resulting extra oxidation of added HdP (Fig. 15), which itself increased oxygen uptake of the extract, as described by van Herk.

The presence of metallo-enzymes in the inflorescence stalk. In sharp contrast to the negative results above, extracts from *Arum* inflorescence stalks gave colour reactions for catechol oxidase, and extracts of a preparation made by the method of Hill & Bhagvat showed active oxidation of the same substrates (Table 8) and (Fig. 14) without prior addition of cytochrome. The oxidation of *p*-phenylenediamine was completely inhibited by M/1000-cyanide (Fig. 16A) and that of catechol by another extract, was strongly inhibited by M/1000-diethyldithiocarbamate (Fig. 16B). Carbon monoxide also induced a strong inhibition of *p*-phenylenediamine oxidation (Fig. 16A), and this was not reversed by light. In view of these results the metallo-enzyme present would appear to be of the polyphenolase type.

DISCUSSION

The experiments reported above lead to some interesting conclusions about the mechanism of the abnormally rapid respiration of the spadix of *Arum* and allied species. The rate is in itself very striking, the Q_{O_2} of tissue slices rising to 31·8 in air and higher in oxygen. Values around 1 are usual in plant tissues, and often show little rise when the tissues are transferred from air to oxygen.

The respiration of most active plant tissues is carried on by means of a metallo-enzyme in the position of terminal oxidase. As a result, it is more or less suppressed by dilute cyanide, and the nature of any residual cyanide-stable respiration is largely a matter of conjecture. The general tendency seems to be for the active respiration of young embryos

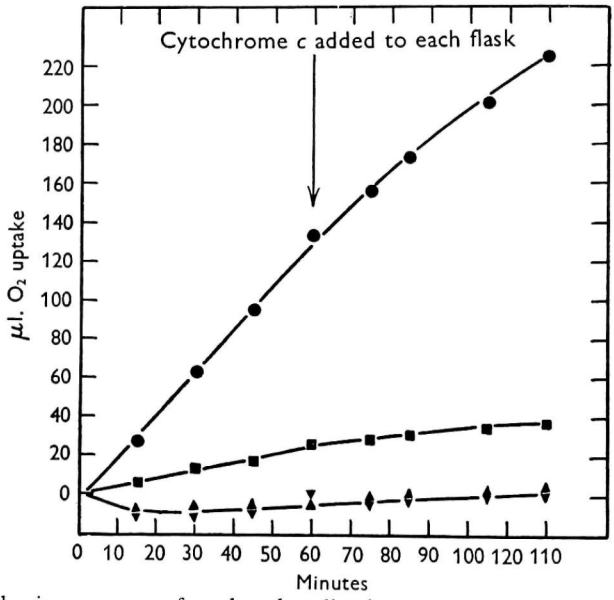

Fig. 14. Oxygen uptake in presence of *p*-phenylenediamine. ●—● Preparation from stalk of *Arum maculatum*; ■—■ the same after boiling; ▼—▼ preparation from spadix; ▲—▲ preparation from spadix with no addition of *p*-phenylenediamine. 0·5 mg. cytochrome-*c* was added to each after 60 min.

(see Merry & Goddard, 1941; Marsh & Goddard, 1939), seedlings (Bottelier, 1939), young leaves (James & Hora, 1940), root tips (James & Harper, unpublished) and the regenerating surfaces of cut disks of storage tissue (Marsh & Goddard, 1939) to be markedly cyanide-sensitive. As the tissues age, and the Q_{O_2} diminishes, it is the cyanide-inhibited respiration that falls off, and the cyanide-stable respiration, without necessarily increasing, becomes a more noticeable fraction of the whole (Marsh & Goddard, 1939).

Van Herk & Badenhuizen (1934) reported that the respiration of *Sauromatum* spadix was inhibited 50% by M/1000 cyanide. This was given as the mean value in a number of experiments not individually reported. Van Herk afterwards (1937) referred to this as a feeble inhibition, a description with which it is not very easy to agree. With the idea that cyanide inhibition might vary with the age of the spadix tissue, we carried out experiments at this concentration with *Arum* at all stages; but in none did the inhibition rise higher than 11% (Table 5), and with four related species (Table 6) the inhibitions were even lower.

Although we are not able to offer an explanation of the relatively high values quoted by van Herk and Badenhuizen for cyanide inhibition, we are able to do so for their similarly

high values with carbon monoxide. They observed a 70% inhibition with a gas mixture of 75% carbon monoxide, 5% oxygen and 20% nitrogen. If cytochrome oxidase, with its very high oxygen affinity, were the operative enzyme, the reduction of oxygen concentration would be without effect on the rate (Green, 1940). With this in mind, presumably,

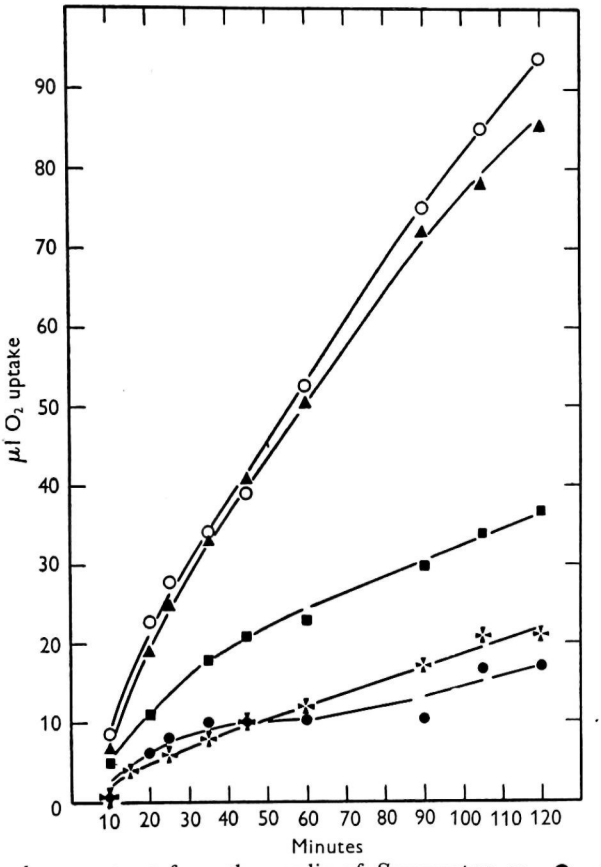

Fig. 15. Oxygen uptake by an extract from the spadix of *Sauromatum* sp. ●—● 2 ml. extract only; ✠—✠ 2 ml. extract + 0·5 ml. M/50-catechol; ■—■ 2 ml. extract + 0·5 ml. M/50-ascorbic acid; ▲—▲ 2 ml. extract + 4 mg. HdP; ○—○ 2 ml. extract + ascorbic acid + HdP.

Table 8. *Oxygen uptake in* μl. *at* pH 7·4 *by* Arum *stalk extracts. All substrates added at* M/100 *concentration*

Substrate	Minutes						
	10	20	30	40	50	80	90
No addition	2	4	2	12	15	25	27
Ascorbic acid	5	5	14	25	27	48	53
p-Phenylenediamine	6	13	26	41	54	79	94
Catechol	24	51	73	92	107	132	139
Hydroxyquinone	4	6	10	17	21	31	35

they ascribed the slowing down of oxygen consumption that they recorded to the presence of the carbon monoxide. Our experiments with slices of *Arum* spadix (p. 361) showed that the oxygen uptake is actually much lower in 4% oxygen than in air; and that, when due allowance is made for this, there is no inhibition by carbon monoxide (Fig. 6). For both these reasons it may be inferred that the cytochrome system plays no part in the spadix

respiration. In addition, we were unable to extract any enzyme that would oxidize *p*-phenylenediamine through cytochrome *c*.

The negative results with cyanide and the 'copper inhibitor' diethyldithiocarbamate further indicate the absence of polyphenolase and ascorbic oxidase; neither could such enzymes be extracted. The conclusion seems clear that no metallo-enzymes are acting as terminal oxidases in the spadix respiration of the aroids we have examined. This is in agreement with the final conclusion of van Herk after his extensive work with *Sauromatum*. It is nevertheless surprising, since, in all previously recorded results, active respiration has always been associated in plant tissues with metallo-enzymes of one kind or another. In this, by far the most active of all, a terminal oxidase that requires a much higher oxygen concentration for saturation is operative. The cytochrome system is saturated at very low oxygen tensions; but the *Arum* spadix respiration was still unsaturated at 100% (1 atm.).

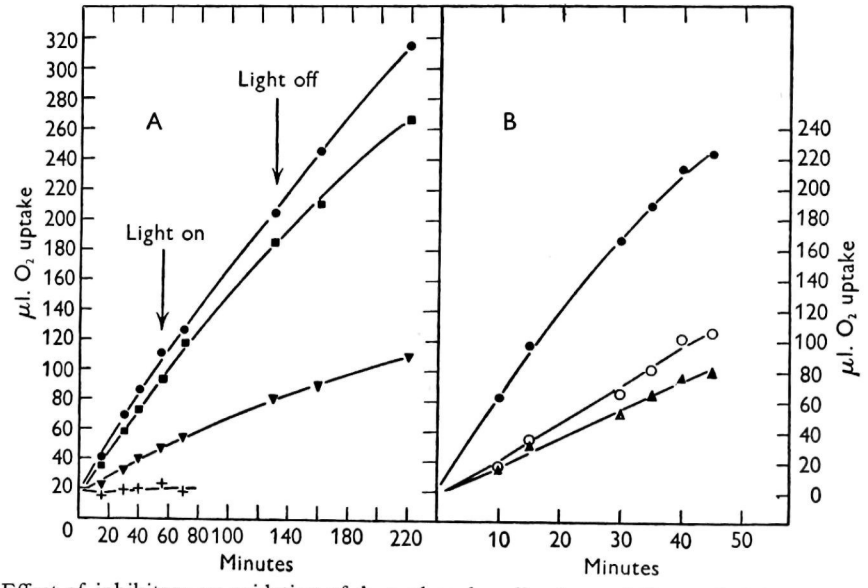

Fig. 16. Effect of inhibitors on oxidation of A, *p*-phenylenediamine and B, catechol by preparation from stalk of *Arum maculatum*. A. ●—● 2 ml. preparation + 0.5 ml. 1% *p*-phenylenediamine in air; ■—■ the same in 5% oxygen + 95% nitrogen; ▼—▼ 5% oxygen + 95% carbon monoxide; +—+ 2 ml. preparation + *p*-phenylenediamine + M/1000-cyanide. B. ●—● 2 ml. preparation + 0·5 ml. M/20-catechol in air; ○—○ the same + M/1000-diethyldithiocarbamate; ▲—▲ the same + M/500-diethyldithiocarbamate.

This recalls the behaviour of flavoprotein systems, and in *Arum* is limited to the spadix itself. The adjacent inflorescence stalk showed oxygen saturation at 5–10% (Fig. 4), yielded an enzyme of polyphenolase type and suffered the appropriate inhibitions.

Glycolysis in the spadix appears to follow conventional lines and to consist of the formation of oxidizable products from hexosediphosphate, since strong inhibitions were observed in dilute fluoride, supposed to be most effective in the reaction phosphoglycerate → phosphopyruvate (Meyerhof & Kiessling, 1935) and in iodoacetate which presumably stopped the formation of phosphoglycerate.

A very unusual feature of the tissue was that it readily yielded crude cell-free extracts which continued both to absorb oxygen and to give off carbon dioxide at high rates. In many respects, such as in its sensitivity to oxygen tension (Figs. 3, 10) and its reactions to the inhibitors cyanide, fluoride and iodoacetate, the behaviour of the

extracted system was remarkably similar to that of the intact tissue, and justifies the view that the complex of respiratory enzymes which is operative *in vivo* had been preserved in an active state. The natural substrates in such crude extracts could be replaced by hexosediphosphate added artificially.

Anaerobic dehydrogenases, capable of using methylene blue as hydrogen acceptor, were also present and were remarkable for their extraordinary activity (p. 366). They were capable of catalysing the oxidation of the natural substrates to complete exhaustion, and of afterwards oxidizing added hexosediphosphate. Van Herk showed that coenzyme I and coenzyme II were both present in the spadix of *Sauromatum* and that hexosediphosphate was oxidized by extracts from this tissue.

The nature of the terminal oxidase, linking the dehydrogenation of the glycolysis products to oxygen, appears to be indicated by the experiments with riboflavin. Additions of riboflavin to the extracted sap caused large increases in the rate of oxygen consumption, which were particularly fast and prolonged in the presence of hexosediphosphate and adenosinetriphosphate. The last may have been of significance in phosphorylating the added riboflavin to form the coenzyme of a flavoprotein. A substance having the properties of a flavoprotein has been extracted from the spadix tissues. When riboflavin was added to an extract containing methylene blue, it no longer caused any acceleration of the oxygen uptake. The methylene blue may thus be regarded as by-passing a terminal flavine enzyme, rather than co-operating serially with it. A further result pointing to a flavoprotein as the terminal oxidase is the low oxygen affinity indicated by the respiration of tissue slices (p. 357) and saps (p. 364). This conclusion is also in agreement with the *Sauromatum* experiments of van Herk, who found unusually large amounts of the flavoprotein enzyme by extraction and estimation as lumiflavin, according to the method of Euler & Dahl (1935). He was not able to show increases of oxygen uptake upon adding flavine to disintegrated tissue such as occurred with our saps.

It is not possible to estimate at present precisely what proportion of the spadix respiration may occur under the influence of the flavoprotein present, and the comparisons of turn-over numbers attempted by van Herk seem to involve too many uncertainties to give information of value. The results obtained by van Herk and by us seem to make it clear that at least a substantial proportion of the spadix respiration may occur by this unusual channel, and that several of the peculiarities of the respiration of aroid spadices may be explained on this account.

Summary

1. The respiration of the spadix of *Arum* species is shown to have Q_{O_2} up to 31·8 in air. There is a progressive increase of respiration rate with increasing oxygen concentration up to 100%. The adjacent stalk tissue has a maximum at 5–10% oxygen.

2. Active gas exchange continued in cell-free extracts with R.Q. approx. 1, and the respiration characteristics of such extracts were closely similar to those of the intact tissue.

3. Oxygen uptake of tissue slices and extracts was not significantly inhibited by M/1000-cyanide at any stage of development of the spadix. Allied species gave the same result.

4. There was no inhibition by carbon monoxide, M/1000-diethyldithiocarbamate, nor by M/20-malonate at pH 3·5.

5. There was marked inhibition with iodoacetate (50% at M/1000 after 4 hr.), and M/500-fluoride at pH 3·5.

6. The oxygen uptake of cell-free extracts was accelerated and prolonged by addition of hexosediphosphate.

7. Decolorization of methylene blue by the extracts was rapid and extensive. By prolonged aeration the contained substrates were eventually depleted, and the power to decolorize methylene blue very much diminished. It was largely restored by adding hexosediphosphate.

8. The oxygen uptake of saps was accelerated by adding riboflavin and adenosinetriphosphate, especially in the presence of hexosediphosphate.

9. The oxygen uptake of saps was strongly accelerated by addition of methylene blue. No further acceleration could then be obtained by adding riboflavin.

10. A substance was obtained in solution from *Arum* spadix tissue which had the properties of a flavoprotein.

11. No cytochrome oxidase, polyphenolase nor ascorbic oxidase could be extracted, although a polyphenolase was extracted from the inflorescence stalk.

12. The available data are taken to indicate that the abnormally rapid respiration rate of aroid spadices involves the breakdown of starch via hexosediphosphate, and that the oxidation stage depends, at least in large part, upon a flavoprotein enzyme. Metalloenzymes play no part.

REFERENCES

BEEVERS, H. & JAMES, W. O. (1948). The behaviour of secondary and tertiary amines in the presence of catechol and belladonna catechol oxidase. *Biochem. J.* **43**, 636.

BEEVERS, H. & SIMON, E. W. (1949). The effect of pH on the activity of some respiratory inhibitors. *Nature, Lond.*, **163**, 408.

BOTTELIER, H. P. (1939). On factors affecting the respiration of the *Avena* coleoptile. *Rec. Trav. bot. néerland.* **36**, 658.

CHURCH, A. H. (1908). *Types of Floral Mechanism.* Oxford.

EULER, H. VON & DAHL, O. (1935). Zur Bildung von Flavinenzym-Systemen in keimenden Pflanzen. *Biochem. Z.* **282**, 235.

GREEN, D. E. (1940). *Mechanisms of Biological Oxidation.* Cambridge.

HERK, A. W. H. VAN (1937). Die chemischen Vorgänge im *Sauromatum* Kolben. *Rec. Trav. bot. néerland.* **34**, 69.

HERK, A. W. H. VAN & BADENHUIZEN, N. P. (1934). Über die Atmung und Katalase-Wirkung im *Sauromatum* Kolben. *Proc. K. Akad. Wet. Amst.* **37**, 99.

HILL, R. & BHAGVAT, K. (1939). Cytochrome oxidase in flowering plants. *Nature, Lond.*, **143**, 726.

JAMES, W. O. & HORA, H. B. (1940). The effect of cyanide on the respiration of barley. *Ann. Bot., Lond.*, N.S., **4**, 107.

KREBS, A. H. (1935). Metabolism of amino-acids. III. Deamination of amino-acids. *Biochem. J.* **29**, 1620.

LATIES, G. G. (1949). Limitations of the use of potassium hydroxide-potassium cyanide mixtures in manometer studies. *J. Biol. Chem.* **177**, 969.

MARSH, P. B. & GODDARD, D. R. (1939). Respiration and fermentation in the carrot, *Daucus carota*. I. Respiration. II. Respiration and the Pasteur effect. *Amer. J. Bot.* **26**, 724, 767.

MERRY, J. & GODDARD, D. R. (1941). A respiratory study of barley grain and seedling. *Proc. Rochester Acad. Sci.* **8**, 28.

MEYERHOF, O. & KIESSLING, W. (1935). Die Umesterungsreaktion der Phosphorbentraubensäure bei der alkoholischen Zuckergärung. *Biochem. Z.* **281**, 249.

TURNER, J. S. (1937). On the relation between respiration and fermentation in yeast and the higher plants. *New Phytol.* **36**, 142.

TURNER, J. S. & HANLY, V. (1947). Malonate and Plant Respiration. *Nature, Lond.*, **160**, 296.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Minneapolis.

WARBURG, O. & CHRISTIAN, W. (1932). Über ein neues Oxydationsferment und sein Absorptionspektrum. *Biochem. Z.* **254**, 438.

WARBURG, O. & CHRISTIAN, W. (1942). Isolierung und Krystallisation des Gärungsferments Enolase. *Biochem. Z.* **310**, 384.