

General Methods of Preparation, Respiratory Gas Analysis and Assessment of Metabolic Pathways

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ABSTRACT: pericarp was measured by direct manometric method and under Warburg conditions. O_2 and CO_2 values were decreased after 60 minutes period as compared to the first by approximately 12% in total cases. In that seedling pericarp restrict, gases exchange that lowered the respiration rate. By removal of the fruit walls the cotyledon tissue is exposed by approximately 75%, acetaldehyde by 87% and ethanol disappears completely, zero levels of fermentation products is observed when pericarp tissue is separated.

KEYWORDS: General methods of preparation, transpiration gases analysis

I. INTRODUCTION

METHODS – TISSUE PREPARATION

Slices, less than 0.5 mm. thick, were hand-cut with a sharp razor from at least 12 cotyledons of medium-sized *Avicennia* or 4 hypocotyls of *Bruguiera*. They were placed immediately in a 1. / (W/v) Kollidor. 25 solution to prevent browning at the cut surfaces caused by phenol oxidation. After a thorough wash in this solution the slices were placed in a small sieve and rinsed in a stream of distilled water for several minutes to remove the contents of out cells. Subsequent activity could then be attributed to undamaged cells.

The slices were then blotted dry on filter paper and randomly selected samples were weighed and placed into 5 m/s. of medium in a Warburg flask. For *Avicennia* 0.5 to 0.8g. and for *Bruguiera* 1.0g. of sliced material were normally used with a suspending medium of distilled water or 0.1M Tris buffer, Ph 6.0 (See appendices I and II for selection of suspending media and effects of Tris buffer on respiration.) Respiratory measurements were started with in 30 minutes of cutting the first slices.

Long term washing techniques have been employed by many workers (e.g. Laties, 1949) to deplete slices of endogenous substrates and so enhance the response to added metabolites. Specific respiratory effects induced by such ageing techniques on mangrove slices are given in appendix III. Apart from washing times procedures were similar to those used for freshly cut slices.

Whole small seedlings of *Avicennia* (1.0 g.) were placed, after careful removal of the pericarp, direct into the flasks either in suspending medium or in a humid atmosphere (0.8 ml. of distilled water in the flask side-arm – see section D)

Leaching and oxidation of phenols occurred if extreme care was not used in pericarp removal. For this reason also (i.e. severe phenolic oxidation), small *Avicennia* seedlings could not be used in tissue slice experiments.

Barley roots approximately 2cm. long were excised from the grain, rinsed thoroughly in distilled water placed intact (0.2 g. of 2 to 3-day old roots) into the manometric flasks.

- Polyvinylpyrrolidone, polymer mt. 25,000-Badische Anilin & Soda-Fabrik AG, Ludwigshafen an Rhein.
- Germination of Barley Grain
- Cape barley (*Hordeum vulgare*) was surface-sterilised in 0.5. / Sodium hypochlorite solution for 10-15 minutes then soaked for 30 minutes in a weaker hypochlorite solution. The grain was suspended in a muslin bag in a

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dark container in well-serated distilled water which was renewed periodically. After 20-24 hours the grain was spread on a moist double sheet of whatman No. 1 paper on a glass- sheet held above a shallow plastic tray. The ends of the paper were allowed to dip into an 0.0025M CaSO_4 solution. Germination was carried out at 25°C for 2-4 days under dark, humid conditions. During growth the roots were kept well –moistened with CaSO_4 . Water is said to give “obviously abnormal “roots (Laties, 1949).

II. SHORT-TERM GAS EXCHANGE MEASUREMENTS

All respiratory measurements were made by the Warburg direct method (Umbreit, Burris & Stauffer, 1957) at 25° c. Following an equilibrium period of 15 minutes readings were taken at 15 to 30 minute intervals for several hours. Respiration rates remained fairly constant over these periods because carbon dioxide concentrations were not allowed to build up to multiplicity of combinations. 10 flask pairs were used to obtain valid comparisons.

In inhibitor experiments the appropriate inhibitors and substrates were made up in distilled water instead of buffer because of possible “deleterious effects of high salt concentrations “(Laties, 1949). There is also the hazard that buffers in undissociated form may penetrate into the cells and alter the sensitivity of the tissue to the inhibitor. Values for oxygen up take or carbon dioxide output were compared with control respiration rates of tissues respiring in water at the same pH. In distilled water, between a pH range of 4 to 6, the endogenous respiration of slices remained constant within narrow limits (Appendix I). An alteration of the pH of the inhibitor solution, however, altered the magnitude of response of the inhibitor solution, however, altered the magnitude of response of the tissue to the inhibitor, showing that the pH effect was associated with the inhibitor not with the pH. In comparisons of carbon dioxide output and ethanol production, allowances were made for any prior exposure of the tissues to the inhibitor before the gas exchange measurements were started. For example, in the case of malonate, tissues were placed in the inhibitor for 60 minutes prior to initial measurements to allow for uniform penetration of the inhibitor and establishment of uniform rates of gaseous exchange (Daly, Sahr & Pazur, 1957.) Tissues were exposed to 2, 4 –dinitrophenol for 30 minutes before the first gas measurement was taken.

In some cases the respiration of tissues was measured in gas atmospheres other than air. Using a manifold system all Warburg flasks in a set could be gassed simultaneously. A rapid gas flow for 30 minutes ensured complete replacement of the previous atmosphere. When a transfer from air to nitrogen was made the 0.2ml. amounts of 15% KOH in the centre- wells were replaced with the same amounts of 0.1N-HCl, and vice versa.

In some experiments, after the gas exchange measurements, flasks were transferred to ice for several minutes before steam distillation of the tissues and liquid flask contents. The presence of inhibitors or substrates did not affect the recoveries of either acetaldehyde or ethanol.

In *Avicennia*, despite the PVP-washing, the slices often tended to discolour, to become flaccid and to leach phenols which became oxidized. Frequently this became too severe for accurate gas measurements to be obtained. Phenol oxidation increased at higher pH values. Addition of PVP to the respiratory medium made little difference to the oxygen uptakes. The problem of phenol oxidation was not severe in *Bruguiera* slices.

III. LONG-TERM MEASUREMENTS OF CARBON DIOXIDE OUTPUT

An automatic pettenkofer mechanism was used to give a continuous record for the evolution of carbon dioxide over periods of time ranging from 10 to 16 days from four plant samples. The automatic mechanism diverted the gas stream through a series of Pettenkofer tubes at fixed intervals. Each day the used tubes were treated in the standard way, then refilled and replaced in the frame ready for the gas stream to come round again. Precautions were taken to prevent carbon dioxide diffusion from the air of the laboratory into the tubes.

To obtain anaerobic atmosphere cylinders of commercial nitrogen* were used. The nitrogen stream was bubbled through strong pyrogallol** to remove any residual oxygen. Strong potassium hydroxide replaced pyrogallol to remove carbon dioxide from the air –steam. As a precaution against contamination the gas was bubbled through a weak aqueous solution of sodium hypochlorite before it entered the plant chambers. This also served to humidify the gas streams. The gas streams were warmed to the temperature of the water-bath (25° c) before entering the respiration chambers. Gas flows were maintained at 25 to 30ml. min^{-1} . Two or three *Bruguiera* seedlings and air to ten

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Avicennia seedlings were used in each respiration chamber. A three hour period of continuous respiration was found to give a more accurate

*Supplied by N.Z. Industrial Gases Ltd.

**75g. Pyrogallol added slowly to 500 mls. KOH (250g.per100ml) measurement of respiration that either one or two hours.

The respiration chambers were rendered light-proof by painting them black and covering them with light-proof paper. The tubes leading to and from the chambers were blackened and the rubber bungs sealing the jars were covered with light-proof black cloth caps. The plant chambers were sterilized and oven-dried before each experiment.

Several workers have found the respiration rate of surface sterilized plant material to be significantly less than that of untreated material and have regarded it as imperative that measurements of respiration should be made under sterile conditions (e.g. Brown, 1942). Denny (1948) found with the majority of seeds he used that the carbon dioxide outputs from those with contaminating micro-organisms was significantly higher than those of sterilized controls.

The pericarp of Avicennia is said to harbor an "extensive micro flora" (Chapman, 1962). Outred (1966) found that it was possible to get an extensive growth of micro-organisms (bacteria and myxomycete colonies) on the surface of agar-plates prepared using water in which intact Avicennia and Bruguiera seedling had been soaking for four days. This growth was suppressed by the incorporation of sterilizing agents into the agar. But increased carbon dioxide outputs due to possible contamination were not significant in mangrove seedlings. In continuous gas stream experiments in both air and nitrogen over eight-day periods, carbon dioxide emissions from samples kept moist with distilled water, sterilizing agent or cloudy supernatant obtained by soaking the seedlings, were closely similar and it was concluded that there was no real need to surface sterilize. However, in view of the long-term nature of some experiments seedlings were soaked for 10 minutes in a sterilizing solution previously determined to be effective in preventing growth on agar-plates. The seedlings were shaken to remove excess moisture and placed under the experimental conditions.

- Miltons antibacterial agent containing 1. / . Sodium hypochlorite, 16.5. / . Sodium chloride.

The chief disadvantage of using the Pettenkofer method is its lack of sensitivity. In later experiments attempts were made to check carbon output results obtained by the Pettenkofer method using an I.R gas analyzer. However, variable results were given and the comparison was abandoned.

IV. LONG- TERM MEASUREMENTS OF METABOLITES

Duplicate seedling samples similar to those used to measure carbon dioxide output were placed in sterilized, light -proof gas jars or conical flasks kept at 25°C and exposed to anaerobic atmospheres in the same way as already described for long-term carbon dioxide measurements. All the seedlings in each group were placed in the same container; the seedlings were loosely packed, by lying the containers lengthwise, to allow ready gas circulation and less chance of localized effects of high, perhaps fluctuating carbon dioxide tensions. Metabolites were analyzed after predetermined time intervals, using, in the case of Avicennia, several seedlings for each analysis, and in the case of Bruguiera, one seedling for each analysis- the latter necessitated by a shortage of material. Seedlings were removed from the plant containers with as little interruption to the gas flow as possible (30 seconds at the most) and either extracted immediately or else removed to air for a short aerobic experience. Samples returned to air before analysis were held in small darkened containers in a temperature-controlled water-bath at 25°C. Samples, except in the large- scale Avicennia experiment (page 300) were extracted at the same time of day to eliminate error due to possible inherent metabolic rhythms in the tissue.

Both Thomas (1925) and Fidler (1934) described acetaldehyde losses occurring by the moving gas stream method. Aqueous solutions of phloroglucinol (1. / .phloroglucinol in 6. / . Sulphuric acid) are described as being suitable for trapping this acetaldehyde. Aqueous acid solutions of phloroglucinol speedily combine with aliphatic aldehydes to produce sparingly soluble substance (phloroglucides) and with an excess of phloroglucinol the amount of precipitation is proportional to the amount of aldehyde present. A comparison of the relative amounts of precipitate formed after the gas stream leading from the plant containers has been bubbled through these solutions should give an estimation of the acetaldehyde loss which is then added on to the total acetaldehyde obtained from steam distillation. However, in mangrove experiments, phloroglucinol inserted in the gas-train sequence immediately after the plant containers gave no

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precipitate, and so this precaution was abandoned. Fidler (1934) also noted that ethyl alcohol could be lost by the continuous gas stream method and that large errors might be introduced if no account was taken of this. The loss of alcohol from apples amounted to 2-15% of the total (Fidler, 1951). Both Fidler (1951) and James and Ritchie (1955) trapped this ethanol in concentrated sulphuric acid. In the present series of experiments no precautions were taken.

Short-term measurements of metabolites:-

In short-term experiments seedling samples (small seedlings or slices) were suspended in small muslin bags in 50 or 100 ml. light proof conical flasks using one plant sample of each container. The tissues were exposed to anaerobic conditions as already described. Samples could be removed rapidly from the experimental conditions and extracted immediately.

The pericarps were removed from *Avicennia* seedlings before each experiment.

Steam Distillation:-

Ethanol, acetaldehyde and lactic acid were extracted by the steam distillation method. In long-term experiments an estimation of "pyruvate" (total keto acids) was made using the lactate fraction. Seedling tissues, removed from the experimental environment, were weighed, cut up if necessary, and transferred to the distillation apparatus immediately. Cut up if necessary, and transferred to the distillation apparatus immediately. It was ensured that this was ready simultaneously with the plant material so that the minimum time lag occurred between the end of the experimental period and the experimental period and the extraction of the respiratory products. Ethanol and acetaldehyde were driven over into an ice-immersed receiving flask, while residues remaining behind in the boiling flask, as well as washings from the tissue pieces, provided a solution containing any lactate and "pyruvate". Large tissue samples were distilled for approximately 20 minutes and smaller. Samples (e.g. barley roots and tissue slices) were distilled for a shorter length of time. It was possible to obtain a quantitative recovery of the desired metabolites (see Appendix VII). The extracts were stored in small, wax-sealed vials at 0°C. Analyses were performed with the minimum delay.

Thomas (1925) used steam distillation to expel alcohol and acetaldehyde from apple tissue, collecting the aqueous distillate in an ice cooled receiving flask and using a second flask in case of any loss from the first. He found that acetaldehyde and alcohol rarely escaped to the second flask, and if they did, then only in trace amounts. A second receiving flask was not used in distillations of mangrove and barley tissues. Fidler (1934) carried out a critical series of tests on the steam distillation technique, measuring the recovery of known quantities of ethanol and acetaldehyde from sliced apple tissue...."Since it was uncertain whether ethyl alcohol and acetaldehyde could be recovered quantitatively from biological material". He came to the conclusion that: "Results show that steam distillation appears to be the best method of recovery of these from plant tissues in a short enough time to preclude secondary changes." The method had been used successfully by a number of workers since (e.g. Siegelman & Hendricke, 1958)

V. LACTIC ACID ESTIMATION

The estimation of lactic acid was carried out using a chemical method based on the quantitative conversion of lactic acid to acetaldehyde by treatment with concentrated sulphuric acid as follows:



The acetaldehyde formed undergoes a purple colour reaction with p-hydroxydiphenyl indicator. The intensity of colour is directly proportional to the amount of acetaldehyde formed. The method used was slightly modified from the micro-method of Hullin and Noble (1953) who found that lactic acid could be determined with an accuracy of $\pm 2\%$ in a protein-free solution.

VI. THE PLANT EXTRACTS WERE TREATED AS FOLLOWS

1.0 ml. of each extract + 1.0 ml. 0.8M copper sulphate solution (i.e. 4.995 g. made to 250 ml.) + 8.0 ml. distilled water + 1.0 g. calcium hydroxide (BDH Technical grade) were mixed in centrifuge tubes sealed with rubber bungs shaken thoroughly and allowed to stand for 20 minutes with occasional shaking thoroughly and allowed to stand for 20 minutes with occasional shaking. This treatment removed any contaminating substances, and in particular pyruvic acid, which would interfere with the colour reaction. The mixture was centrifuged and 1.0 ml. of the clear supernatant pipette into a quickfit pyrex tube, with a ground glass stopper, immersed in ice. To this was slowly added 8.0 ml. chilled

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concentrated sulphuric acids (Analar) +0.05 ml. 5. /. Copper sulphate solution. A slight excess of copper sulphate made no difference to the final result. The tube were placed in a water-bath at $60^{\circ}\text{C} - 1^{\circ}$ for 30 minutes, then cooled to $10-15^{\circ}\text{C}$. Stoppers were removed and 0.20ml. p-hydroxydiphenyl indicator was added close to the surface, rotating gently to mix thoroughly the precipitated p-hydroxydiphenyl. Adequate cooling of the indicator on its addition. A slight excess of indicator made no difference to the final result (Barker, 1957). The tubes were placed in a water-bath at $30^{\circ}\text{C} - 1^{\circ}$ for 30 minutes. This length of incubation was allowed to ensure maximum colour development but longer heating time did not affect the results. Following this, the tubes were placed in boiling water for 90 seconds to destroy excess p-hydroxydiphenyl and to stabilize the colour. The heating time was not critical up to 2 minutes (Barker, 1957). The tubes were cooled immediately in ice-water and estimated at a wave-length peak of $560\text{m}\mu$ against a reagent blank prepared by taking distilled water through the whole of the above procedure. Barker found that the colour diminished by 5. /. after 3 hours at room temperature.

p-hydroxydiphenyl indicator was made by dissolving 1.0g. in hot 2N-NaOH (25ml.) and adding 75ml. distilled water before cooling. The indicator was stored in a dark bottle in the refrigerator. It is stable indefinitely unless precipitation or discoloration occurs.

The method was critically investigated by Barker (1957), who found that conditions under which the estimations were carried out must be carefully standardized. Unless very stringent precautions were taken highly variable results were obtained from replicate estimations of aliquots from the same sample. In particular, the conditions under which the oxidation of lactic acid to acetaldehyde was carried out, in the first stages of the estimation, were of critical importance. When carried out in open tubes, the acetaldehyde recoverable as a final coloured complex varied greatly with the heating employed. The glassware used must be clean, oven-dried and stored in dust-free conditions.

Clausen (1922) found that many other substances, especially other α -hydroxy acids, yielded aldehydes and ketones on hot acid treatment and also that many substances reacted with acetaldehyde in the presence of acid. For example, 1 mg. of phenol added to 1mg. of lactic acid was found to decrease the yield of acetaldehyde by more than 50 /. Calusen removed interfering compounds in the extracts using Plaster of Paris. Calcium hydroxide has also been used to remove glucose and other interfering or reacting substances. Barker (1957) found that several compounds were not removed for example, methionine, which reduced the colour with p-hydroxydiphenyl. This is not likely to be present in steam-distilled extracts. It was necessary to treat mangrove extracts with calcium hydroxide, otherwise the dark colour which developed on addition of acid completely masked the purple colour reaction.

The amount of lactic acid in each sample was calculated by comparison with calibration graphs which were prepared for each series of determinations and which were linear over the range 0 to 80ug. ml^{-1} (fig .6) A calibration graph was built up by weighing an exact amount of Analar lactic acid(0.8g.) into a weighing bottle. This was made to 100mls. And 10mls. Of this stock solution made to 1 litre. A dilution series built up from this gave a range of concentration from 8 to 80ug. ml^{-1} . Provided all the precautions outlined were taken, duplicate determinations on lactic acid samples over this range agreed to within 3%

The possible presence of inhibitors of the assay system in the tissue extract was investigated by estimating a given level of standard and extract separately and together in the same assay and comparing the expected and the observed result in a combined assay. Recoveries ranged from 96 /. to 104/.(table 4).

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Table . Recoveries of standard lactate samples added to tissue extracts.

	Lactate (ug.ml. ⁻¹)		
In sample originally	23.88	23.91	39.59
Added lactated	19.64	46.97	19.64
Total recovered	44.00	68.80	59.24
Recovery of added lactate	20.12	44.89	19.65
%Recovery of added lactate	103.56	95.75	100.00

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1. Laties, (1949) to deplet slices of endogenous substrates and so enhance the response to added metabolites
2. Umbreit, Burris & stauffer,(1957) at 25° c. Following an equilibrium period of 15 minutes readings were taken at 15to 30 minute intervals for several hours.
3. Brown, (1942). Denny (1948) found with the majority of seeds he used that the carbon dioxide outputs from those with contaminating micro-organisms was significantly higher than those of sterilized controls.
4. Chapman,(1962). Outred (1966) found that it was possible to get an extensive growth of micro-organisms (bacteria and myxomycete colonies)
5. Both Thomas (1925) and Fidler (1934) described acetaldehyde losses occurring by the moving gas stream method.
6. Fidler (1934) also noted that ethyl alcohol could by lost by the continuous gas stream method and that large errors might be introduce d if no account was taken of this
7. Both Fidler (1951) and James and Ritchie (1955) trapped this ethanol in concentrated sulphuric acid. In the present series of experiments no precautions were taken.
8. Clausen (1922) found that many other substances, especially other –hydroxy acids, yielded aldehydes an ketones on hot acid treatment and also that many substances reacted with acetaldehyde in the presence of acid.