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FUNGAL METABOLISM—II.

STUDIES ON THE FORMATION AND ACTIVITY OF *p*-DIPHENOL OXIDASE (LACCASE)

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Abstract—A great increase in the activity of extracellular *p*-diphenol oxidase was found to occur in stationary cultures of *Polyporus versicolor* as compared with shaken cultures. The addition of copper sulphate to the growth medium increased the enzymic activity. The oxidation of 2,6-dimethoxyphenol was studied by a spectrophotometric method. The presence of an initial induction phase was noted. The induction period was eliminated by the presence of the quinonoid product and prolonged by the presence of ascorbic acid and ferrous ions. Ascorbic acid was found to be a substrate of the enzyme. The K_m for 2,6-dimethoxyphenol and 2,6-dimethylphenol were obtained. A study of the effect of pH on the oxidation of 2,6-dimethoxyphenol showed a broad pH optimum below pH 3·8.

INTRODUCTION

EARLY reports on the substrate specificity of *p*-diphenol oxidase (Laccase, *p*-diphenol:O_2 oxidoreductase*) by Keilin and Mann[1] and Gregg and Nelson[2] claimed that *p*-diphenol oxidase was inactive in the oxidation of monohydroxyphenols. More recently, studies by Fåhraeus and Ljunggren[3] using purified *p*-diphenol oxidase from *Polyporus versicolor* showed that under the appropriate conditions *p*-cresol and other monohydroxyphenols were oxidized by this enzyme. The products obtained when *p*-cresol was oxidized by *p*-diphenol oxidase were later identified by Benfield *et al.*[4] At present few quantitative studies have been reported on the oxidations catalysed by *p*-diphenol oxidase compared with numerous studies on *o*-diphenol oxidase (tyrosinase) from many sources.[5]

RESULTS AND DISCUSSION

In the studies reported here, a monohydroxyphenol (2,6-dimethoxyphenol) was used as substrate in the spectrophotometric assay of the enzyme. The product of the reaction, 3,5,3′,5′-tetramethoxydiphenoquinone, is, when pure, extremely insoluble in water and the usual organic solvents, but is somewhat soluble in phenols and therefore remains in solution in the presence of 2,6-dimethoxyphenol, and crystallizes out of the incubation mixture when the solution becomes saturated. The disadvantages of the assay are due to the insolubility of purified 3,5,3′,5′-tetramethoxydiphenoquinone, which although soluble to some extent in chloroform, rapidly loses its colour in this solvent.

* Nomenclature designated by the I.U.B. Commission on Enzymes (*Report of the Commission on Enzymes of the I.U.B.*, p. 81. Pergamon Press, Oxford (1961)).

[1] D. KEILIN and T. MANN, *Nature* **143**, 23 (1939).
[2] D. C. GREGG and J. M. NELSON, *J. Am. Chem. Soc.* **62**, 2500 (1940a).
[3] G. FÅHRAEUS and H. LJUNGGREN, *Biochim. Biophys. Acta* **46**, 22 (1961).
[4] G. BENFIELD, SHEILA M. BOCKS, K. BROMLEY and B. R. BROWN, *Phytochem.* **3**, 79 (1964).
[5] D. KERTESZ and R. ZITO, In *Oxygenases* (Edited by O. HAYAISHI), p. 307. Academic Press, New York (1962).

The enzyme is liberated into the growth medium and the increase in enzymic activity of the medium was studied in both stationary and shaken cultures during growth of the fungus. The results are represented in Fig. 1. In shaken cultures, the activity of the medium reached a maximum level after approximately 9 days, this maximum was maintained for about 6 days and then the activity began to decline. In stationary cultures, the enzymic activity of the growth medium continued to increase for approximately 24 days and then began to decline. The enzymic activity reached a much higher maximum in stationary cultures. Enzymic activity was not detected in the growth medium of stationary cultures left for periods of 8–12 weeks. *Polyporus versicolor* belongs to the group of wood-rotting Basidiomycetes and this enzyme has been associated with the oxidation of phenolic constituents of wood. The pattern of enzymic activity found in the stationary cultures may resemble that occurring under

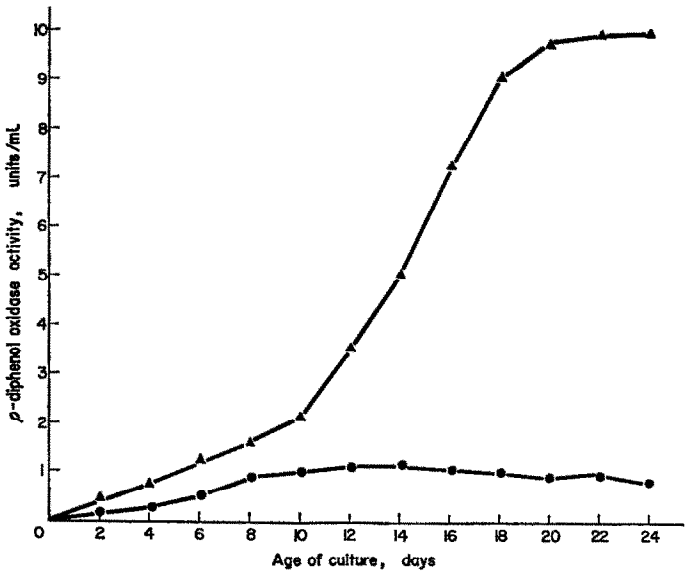


FIG. 1. *p*-DIPHENOL OXIDASE ACTIVITY OF *P. versicolor* CULTURES. THE ENZYMIC ACTIVITY OF ALIQUOTS OF CULTURE MEDIUM WERE ASSAYED AT INTERVALS BY THE METHOD DESCRIBED UNDER "EXPERIMENTAL". ▲, Stationary cultures; ●, shaken cultures.

natural conditions and is associated with the early phases of the establishment of the fungus, since the enzyme was not detected in the medium of "old" cultures.

p-Diphenol oxidase of *P. versicolor* is a copper-containing enzyme[6] and to determine whether copper was a limiting factor in the formation of the enzyme, copper in the form of copper sulphate was added to the growth medium in varying concentrations. The results showed that the enzymic activity was increased by addition of copper to the medium. At the highest level of copper used (250 μg/ml), the growth of the organism was severely inhibited and enzymic activity was not detected. The increase in enzymic activity could not be attributed to the activity of copper complexes formed with constituents of the growth medium or to the ionic copper itself, since no activity was detected in un-inoculated cultures or boiled samples of the media from the cultures. Experiments were also carried out to test the effect of copper sulphate on the enzymic acitivity of culture filtrates of the organism grown in the absence of

[6] L. BROMAN, B. G. MALSTRÖM, R. AASA and T. VANNGARD, *Biochim. Biophys. Acta* **75**, 365 (1965).

added copper sulphate. The final concentrations of copper sulphate used in the spectrophotometric assay were 1 μg, 10 μg, 100 μg, 500 μg and 1000 μg. No increase in *p*-diphenol oxidase activity was observed, but at concentrations of 100 μg and above a slight inhibition of enzymic activity was obtained. Hence the increase in enzymic activity (Table 1) appears to represent a stimulation of enzyme production.

TABLE 1. THE EFFECT OF Cu^{++} CONCENTRATION ON THE GROWTH OF *P. versicolor* AND *p*-DIPHENOL OXIDASE ACTIVITY OF THE CULTURE FLUID AFTER 2 WEEKS' INCUBATION AT 25° AS STATIONARY CULTURES

Cu^{++} concentration (μg/ml)	Growth (mycelial dry wt., g)	*p*-Diphenol oxidase activity (units/ml)
0	1·4863	2
2·5	1·4891	4·54
25	1·4769	4·8
250	0·0452	0·05

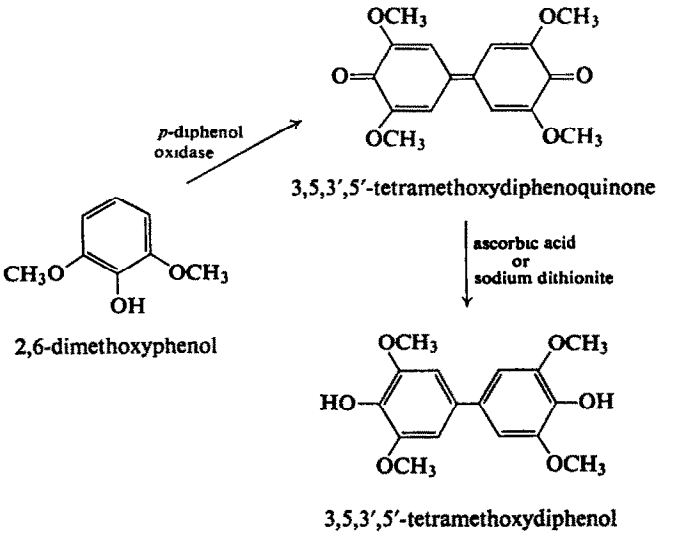
When the oxidation of 2,6-dimethoxyphenol to 3,5,3′,5′-tetramethoxydiphenoquinone was catalysed by *p*-diphenol oxidase under the conditions described (see Experimental), a short induction period (1–2 min) was always observed. This phase is of too short a duration to be noted when the activity of the enzyme is measured by the manometric technique. It was soon apparent that if solutions of substrate were left to undergo autoxidation to a pale straw colour at room temperature for a few weeks before use, no induction period was observed. The straw colour of "old" solutions of substrate was shown to be due to the presence of 3,5,3′,5′-tetramethoxydiphenoquinone, by extracting the acidified solutions of substrate into chloroform, the u.v. spectra of the chloroform extracts were identical with those of the authentic compound. It was necessary to use fresh preparations of the substrate in all kinetic studies to obtain consistent results. There is at present no satisfactory explanation regarding the mechanism by which the quinonoid product functions in eliminating the lag period. The lag may represent the time taken for the building up of a certain concentration of the product after which the rate of oxidation becomes linear. This induction period resembles the pre-steady state phase studied by Gutfreund,[7] where a short time-interval occurs during which the enzyme-substrate complex is building up and the reaction is accelerating. However, in the case of the enzymes studied by Gutfreund, the presteady state was very short and measured in milliseconds by special methods. Complete lack of activity was observed in controls containing (*a*) boiled enzyme and (*b*) boiled enzyme and 5 μmoles cupric ion in the form of $CuSO_4.5H_2O$, so that the activity cannot be attributed to the presence of extraneous copper.

Since the induction period was eliminated by the presence of the product, it was concluded that any substance which might reduce the quinone to the corresponding phenol or prevent the formation of the quinone, may increase the length of the initial induction period. This was found to be so with ascorbic acid and ferrous ions, neither of which caused a significant change in the final rate, only an increase in the length of the induction period, with increasing concentrations. Thus with 1·0 μmole of either the induction period was approximately 6–7 min.

Ferrous ions and ascorbic acid were found to reduce 3,5,3′,5′-tetramethoxydiphenoquinone to the corresponding phenol when added to incubation mixtures containing active

[7] H. GUTFREUND, *Discussions Faraday Soc.* No. 20, p. 167 (1955).

enzyme and substrate, after oxidation had been allowed to proceed for some time. Reduction of the quinone was very rapid with ascorbic acid and much slower with ferrous ions. Examination of the products by thin-layer chromatography showed the presence of a compound with R_f and colour reactions identical to 3,5,3′,5′-tetramethoxydiphenol prepared by the reduction of 3,5,3′,5′-tetramethoxydiphenoquinone by sodium dithionite. However, in experiments in which excess ascorbic acid was added initially no reduced quinone could be detected by these methods.



The lag produced in the presence of ferrous ions and ascorbic acid is presumed to be due to the reduction of the quinone product. The first stage may be represented by reaction (1) and the quinone formed may then be reduced to the corresponding diphenol (2), which could then be reoxidized according to reaction (3). However, in the case of ascorbic acid a further reaction may occur (4), since ascorbic acid itself was found to be oxidized by the enzyme (Fig. 2) in experiments using conventional manometry to measure the rate of oxygen uptake during the oxidation of ascorbic acid.

$$\text{2,6-dimethoxyphenol} \xrightarrow[\text{enzyme}]{O_2} \text{3,5,3′,5′-tetramethoxydiphenoquinone} \quad (1)$$

$$\text{3,5,3′,5′-tetramethoxydiphenoquinone} \xrightarrow[\text{or ascorbic acid}]{Fe^{2+}} \text{3,5,3′,5′-tetramethoxydiphenol} \quad (2)$$

$$\text{3,5,3′,5′-tetramethoxydiphenol} \xrightarrow[\text{enzyme}]{O_2} \text{3,5,3′,5′-tetramethoxydiphenoquinone} \quad (3)$$

$$\text{ascorbic acid} \xrightarrow[\text{enzyme}]{O_2} \text{dehydroascorbic acid} \quad (4)$$

Evidence for the oxidation of ascorbic acid by *p*-diphenol oxidase from other sources has been presented before. The earliest reports of the ability of the enzyme to catalyse the oxidation of ascorbic acid were those of Bertrand,[8] he suggested that in many plant tissues the

[8] D. BERTRAND, *Bull. Soc. Chim. Biol.* **27**, 396 (1945); *Compt. Rend.* **221**, 35 (1945).

enzyme that oxidizes ascorbic acid is probably laccase (*p*-diphenol oxidase) rather than ascorbic acid oxidase. Thus laccase could function as a terminal oxidase in these plants. Later, Omura,[9] using purified enzyme from *Rhus vernicifera* and *R. succedanea*, found that ascorbic acid was a substrate for both enzymes. More recently, Peisach and Levene,[10] using purified enzyme from *R. vernicifera* and ceruloplasmin, also found that ascorbic acid served as a substrate for both enzymes. Ceruloplasmin is a copper oxidase present in serum and has often been described as an animal laccase. The direct oxidation of ascorbic acid by ceruloplasmin is of particular interest since evidence for ascorbic acid oxidase in animal tissues has not been presented, but it has been suggested that the oxidation of L-ascorbic acid in animal tissues may be coupled with an electron transport system involving cytochrome oxidase.[11]

Reaction (2) is also known to occur during the oxidation of phenols, catalysed by *o*-diphenol oxidase (tyrosinase) in the presence of ascorbic acid, but there are considerable

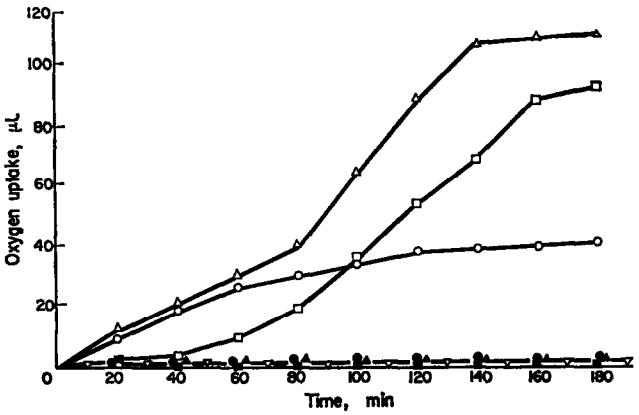


FIG. 2. OXYGEN CONSUMPTION DURING THE OXIDATION OF ASCORBIC ACID AND 2,6-DIMETHOXYPHENOL CATALYSED BY *p*-DIPHENOL OXIDASE.

△, Ascorbic acid (5 µmoles) and 2,6-dimethoxyphenol (3 µmoles); ○, 2,6-dimethoxyphenol (3 µmoles); □, ascorbic acid (5 µmoles); ▽, control, enzyme and buffer only; ■, control, ascorbic acid (5 µmoles), minus enzyme; ●, control, 2,6-dimethoxyphenol (3 µmoles), minus enzyme; ▲, control, 2,6-dimethoxyphenol (3 µmoles) and ascorbic acid (5 µmoles), minus enzyme. (Details under "Experimental".)

differences between the types of phenol oxidases. Apart from differences in substrate specificity, *p*-diphenol oxidases from all sources have been shown to be blue copper proteins in which at least some of the copper atoms are present in the divalent state[12–14] and a valency change (Cu^{2+}–Cu^{1+}) occurs during oxidation of the substrates. On the other hand, purified *o*-diphenol oxidases from all sources so far described are colourless enzymes[5,15] containing monovalent copper only. Kertesz,[16] using a chemical method, has shown that no valency change occurs during oxidation of the substrates by *o*-diphenol oxidase. Hence the mechanism of oxidation catalysed by the two types of enzymes may be very different.

[9] T. OMURA, *J. Biochem.* (*Tokyo*) **50**, 264 (1961).
[10] J. PEISACH and W. G. LEVENE, *J. Biol. Chem.* **240**, 2284 (1966).
[11] W. KERSTEN, H. SCHMIDT and H. STAUDINGER, *Biochem. Z.* **326**, 469 (1955).
[12] B. G. MALSTRÖM, R. MOSBACH and T. VANNGARD, *Nature* **183**, 321 (1959).
[13] T. NAKAMURA, *Biochim. Biophys. Acta* **42**, 477 (1960).
[14] A. EHRENBERG, B. G. MALSTRÖM, L. BROMAN and R. MOSBACH, *J. Mol. Biol.* **5**, 450 (1962).
[15] M. FLING, N. H. HOROWITZ and S. F. HEINNEMAN, *J. Biol. Chem.* **328**, 2045 (1963).
[16] D. KERTESZ, *Nature* **180**, 506 (1957).

Addition of ferric ions in the form of ferric chloride (0·5 μmole) and ferric ammonium sulphate (0·05 μmole) resulted in the oxidation of some 2,6-dimethoxyphenol to 3,5,3′,5′-tetramethoxydiphenoquinone, in addition, brownish coloured complexes of ferric ion and the substrate were formed before the addition of enzyme. After addition of the enzyme, inhibition of the enzymic activity was obtained but the results were erratic due to the slight turbidity produced in the incubation mixtures owing to the presence of the complexes. A meaningful interpretation of the inhibitory effect would only be possible when more is known of the many reactions taking place in the presence of ferric ions under the conditions used. A study of the effect of pH on the oxidation of 2,6-dimethoxyphenol with three buffers showed a broad pH optimum range on the acid side below pH 3·8 (Fig. 3). Slight variations occur with the buffers used and the initial induction phase was found to increase as the pH was increased above pH 4·0. The *p*-diphenol oxidase from *P. versicolor* is produced as an

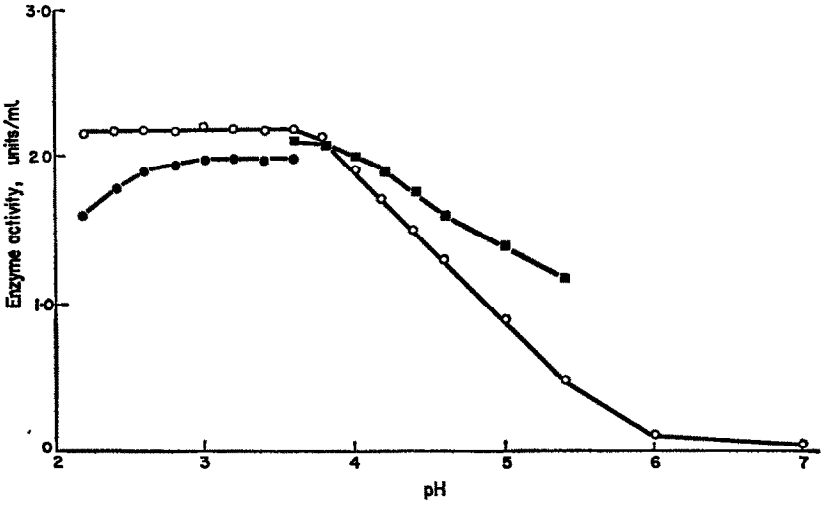


FIG. 3. THE EFFECT OF pH ON THE OXIDATION OF 2,6-DIMETHOXYPHENOL BY *p*-DIPHENOL OXIDASE.
●, Glycine–HCl buffer; ■, sodium acetate–acetic acid buffer; ○, disodium hydrogen phosphate–citric acid buffer. The concentration of enzyme was 2·1 units.

extracellular enzyme and the acidic pH optimum may reflect an adaptation to the environment in which this organism is normally found. The enzyme displayed Michaelis–Menton kinetics with 2,6-dimethoxyphenol as substrate, reciprocal plots of initial velocity and substrate concentration gave straight lines. The K_m for 2,6-dimethoxyphenol at pH 3·6 was found to be $3{\cdot}2 \times 10^{-5}$ M.

The oxidation of 2,6-dimethylphenol was also studied by the spectrophotometric method. The product obtained was 3,5,3′,5′-tetramethyldiphenoquinone.[4] The oxidation of 2,6-dimethylphenol showed differences from that of 2,6-dimethoxyphenol. A much longer initial induction period occurred before a steady state was reached. The lag lasted for approximately 4 min when substrate concentrations of 15–30 μmoles were used. The K_m for 2,6-dimethoxyphenol was $2{\cdot}5 \times 10^{-3}$ M.

The increase in the initial lag and the higher K_m value suggests that 2,6-dimethylphenol has a lower affinity for the enzyme. This conclusion is supported by the work of Peisach and Levene[10] on ceruloplasmin and *p*-diphenol oxidase from *R. vernicifera*. They suggested that

two *ortho*- or *para*-related electron supplying groups are necessary on the aromatic substrate for best activity, while electron-withdrawing groups were found to decrease activity. Thus the difference in electron-donating effect of the O-methyl and methyl groups appears to be reflected in the activity of 2,6-dimethoxyphenol and 2,6-dimethylphenol as substrates.

EXPERIMENTAL

The organism *Polyporus versicolor* was maintained on agar slopes containing 3 per cent malt extract. Liquid cultures of the organism were grown under sterile conditions, in 250-ml conical flasks containing 150 ml aqueous malt extract (3% w/v), as surface cultures supported on glass wool. $CuSO_4 \cdot 5H_2O$ was added to the medium before sterilization to give a final concentration of 2·5 μg Cu^{++} per ml. The cultures were incubated at 25°. Shaken cultures were incubated on a gyrotory shaker (80 rev/min) at the same temperature. The wild strain of *P. versicolor* used was kindly provided by Mr. J. Keeping of this laboratory. The preparation of *p*-diphenol oxidase used in the kinetic studies was carried out according to the method previously described by Malström *et al.*[17]

Determination of p-*Diphenol Oxidase Activity*

The enzymic activity was measured spectrophotometrically, 1·0 unit of enzyme caused a change of extinction of 1/min, at 468 nm, the λ_{max} of 3,5,3′,5′-tetramethoxydiphenoquinone. The initial velocity of the reaction at 25° was measured from the linear portion of the curve.

The incubation mxiture consisted of 3·24 μmoles, 2,6-dimethoxyphenol, and sodium acetate buffer, pH 3·6 (2·4 μmoles), in a final volume of 3·5 ml, in a 1-cm glass cell. Any additions to the incubation mixture are mentioned in the legends to the figures. The enzyme solution (100 μl) was added to the reaction cell after the incubation mixture had reached thermal equilibrium and the solution was thoroughly mixed. A Unicam S.P. 600 spectrophotometer fitted with a thermostatically controlled cell holder was used. A reagent blank and adequate controls were used in all experiments.

In kinetic studies of the oxidation of 2,6-dimethylphenol a range of substrate concentrations from 5 μmoles to 33 μmoles were used and the enzyme concentration was doubled, but the final volumes used were the same as for 2,6-dimethoxyphenol. The increase of absorbancy was measured at 422 nm the λ_{max} of 3,5,3′,5′-tetramethyldiphenoquinone.

Ferrous sulphate, ferric chloride, ferric ammonium sulphate, copper sulphate and the chemicals used in the preparation of buffer solutions were of Analar grade. Ascorbic acid, 2,6-dimethoxyphenol and 2,6-dimethylphenol were obtained from Koch-Light Laboratories, Colnbrook, Bucks. Buffer solutions were prepared as described by Dawson *et al.*[18], except for the sodium acetate–acetic acid buffer which was used at a final concentration of 2·4 μmoles per 3·5 ml as higher concentrations were found to be inhibitory.

Thin-layer chromatography of phenolic compounds was carried out on cellulose plates using 2% acetic acid and butanol–ethanol–ammonia (4:1:5, by vol.) (top-layer) as solvents. The compounds were located by spraying with (i) diazotized-*p*-nitroaniline and (ii) 5% (w/v) ferric chloride and 3% (w/v) potassium ferricyanide mixed in equal volumes just before use. Oxygen uptake was measured by the conventional manometric technique. 2,6-Dimethoxyphenol (3·0 μmoles) and ascorbic acid (5 μmoles) were used separately and as mixed substrates. Control experiments (minus enzyme) showed that no measurable oxygen uptake occurred due to autoxidation of the substrates. Each manometer flask also contained sodium acetate buffer, pH 4·0 (2·4 μmoles), and 0·5 units of enzyme, any carbon dioxide present was absorbed by KOH in the centre well. The experiments were carried out at 30°.

[17] B. Malström, G. Fåhraeus and R. Mosbach, *Biochim. Biophys. Acta* **28**, 652 (1958).
[18] In *Data for Biochemical Research* (Edited by R. M. C. Dawson, D. C. Elliot, W. H. Elliot and K. M. Jones), p. 195. Clarendon Press, Oxford (1959).