Resolution of the Methane Mono-oxygenase of *Methylococcus capsulatus* (Bath) into Three Components

PURIFICATION AND PROPERTIES OF COMPONENT C, A FLAVOPROTEIN

By JOHN COLBY and HOWARD DALTON

Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, U.K.

(Received 15 September 1977)

1. Ion-exchange chromatography resolves the methane mono-oxygenase from soluble extracts of *Methylococcus capsulatus* (Bath) into three fractions. 2. Fractions A and B are comparatively stable at 0°C, whereas fraction C is very unstable unless kept in the presence of sodium thioglycollate (1–10 mM) or dithiothreitol (5–10 mM). 3. The active component from fraction C was purified some 80-fold. 4. Purified component C has mol.wt. 42000. Its solutions are yellow with absorption maxima at 270 and 465 nm and a shoulder at 395 nm. The 465 nm peak is abolished by reduction with NADH or sodium dithionite, or by photoreduction in the presence of EDTA. A new spectral species, probably a neutral flavin semiquinone, is observed on partial reduction of component C. 5. No copper was detected in samples of purified component C, but the protein contains 1.3–1.5 atoms of iron/molecule. 6. On boiling, component C releases a yellow–green fluorescent material that has been identified as FAD from its absorption and fluorescence spectra and by t.l.c. 7. Component C contains 1 mol of FAD/mol of protein.

The methane mono-oxygenase present in soluble extracts of *Methylococcus capsulatus* (Bath) is a very non-specific enzyme capable of oxidizing a variety of alkanes, alkenes, ethers and cyclic compounds (Colby *et al.*, 1977) as well as ammonia (Dalton, 1977) with NAD(P)H as electron donor. It has previously been shown to be a multi-component enzyme system (Colby & Dalton, 1976), and this has now been confirmed by the resolution of the enzyme activity into three fractions. The present paper describes the purification and properties of one component, component C, which is a metalloflavo-protein.

Materials and Methods

Materials

Ethene was from Cambrian Chemicals, Croydon, Surrey, U.K. DEAE-cellulose (exchange capacity 0.9 mequiv./g), Sephadex G-75, Sephadex G-200, sodium thioglycollate, dithiothreitol, 2-mercapto-ethanol, L-cysteine hydrochloride, reduced glutathione, FAD, FMN, riboflavin, sodium L-ascorbate, spermine tetrahydrochloride, crystalline bovine plasma albumin, ox liver catalase, ovalbumin, yeast alcohol dehydrogenase, α -chymotrypsinogen A, *Escherichia coli* alkaline phosphatase, pig heart malate dehydrogenase, horse heart myoglobin, deoxyribonuclease I and 2,9-dimethyl-1,10-phenanthroline (neocuproine) were from Sigma (London)

Chemical Co., Kingston upon Thames, Surrey, U.K. NADH (grade 2) was from Boehringer Corp. (London), London W. 5, U.K., and ethanol-free solutions were prepared as described previously (Colby *et al.*, 1977). BDH Chemicals, Poole, Dorset, U.K., supplied 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline), and Anderman and Co., East Molesey, Surrey, U.K., supplied Merck t.l.c. glass plates coated with silica gel H₂₅₄.

Growth of *M. capsulatus* (Bath) and preparation of soluble extracts

This was done as described previously (Colby *et al.*, 1977) except that the washed organisms were resuspended in 20 mM-Tris/HCl buffer, pH 7, containing 5 mM-MgCl₂ and deoxyribonuclease I (0.01 mg/ml) before breakage in the French pressure cell at 137 MPa (20000 lb/in²).

Separation of methane mono-oxygenase activity into three fractions

Soluble extract (120 ml) was thawed and placed on a 4.5 cm × 12 cm column of DEAE-cellulose equilibrated with 20 mM-Tris/HCl buffer, pH 7. The material not binding to the column packing (fraction A) was eluted with 20 mM-Tris/HCl buffer, pH 7, and immediately frozen by dropwise addition to liquid N₂. The column was then eluted with successive 500 ml batches of the same buffer containing respectively 0.1 M-, 0.2 M- and 0.5 M-NaCl. The 0.1 M-NaCl

eluate was discarded, but the highly coloured fractions from the 0.2M-NaCl eluate were combined (fraction B) and frozen by dropwise addition to liquid N₂. The highly coloured fractions from the 0.5M-NaCl eluate (fraction C) were treated similarly.

Purification of component C

All procedures were carried out at 0–4°C and the buffer used throughout was 20mM-Tris/HCl, pH7, containing 5mM-sodium thioglycollate. The same buffer was used to equilibrate all chromatography columns before use.

Step 1: (NH₄)₂SO₄ fractionation. Fraction C (usually 60–70ml) was thawed and sufficient 1M-sodium thioglycollate added to make the final concentration 5mM. (NH₄)₂SO₄ was added slowly with stirring until the solution was 45% saturated. The suspension was centrifuged at 35000g for 10min and the precipitate discarded. The supernatant was then adjusted to 65% saturation by the addition of more (NH₄)₂SO₄. The precipitate was collected by centrifugation as above, redissolved in about 4ml of buffer and then dialysed against 1 litre of buffer for 2h to remove the (NH₄)₂SO₄.

Step 2: Sephadex G-75 chromatography. The dialysed (NH₄)₂SO₄ fraction was applied to a 90cm×3.8cm column of Sephadex G-75 and eluted with buffer at a flow rate of 40ml/h. The active fractions from the column were combined.

Step 3: DEAE-cellulose chromatography. The combined active fractions from step 2 were placed on a 1cm×12cm column of DEAE-cellulose and eluted with a linear gradient (total volume 500ml) of 0–0.5 M-NaCl in buffer. The active fractions from the column were combined and then concentrated to 1.5ml in a 10ml ultrafiltration cell (Amicon, High Wycombe, Bucks., U.K.) over an Amicon PM10 membrane.

Step 4: Sephadex G-200 chromatography. The material from step 3 was applied to the top of a 90cm×1cm column of Sephadex G-200 and eluted with buffer at a flow rate of 7ml/h. The active fractions were combined and concentrated by ultrafiltration over a PM10 membrane before freezing in liquid N₂.

Enzyme assays

Methane mono-oxygenase activity was determined as described by Colby *et al.* (1977) by using ethene (134 μmol) as substrate and measuring epoxyethane formation. Ethene is oxidized more rapidly than methane and is the preferred substrate because the product of its oxidation, epoxyethane, is not susceptible to further oxidation by the mono-oxygenase.

Protein

Protein concentrations were determined with the Folin–Ciocalteu reagent (Kennedy & Fewson, 1968)

with dried crystalline bovine plasma albumin as standard.

Polyacrylamide-gel electrophoresis

The purity of the purified component C was determined by two different electrophoretic methods. (1) Untreated protein (1 mg/ml) in 6% (w/v) sucrose was loaded on non-denaturing cylindrical 10% (w/v) acrylamide gels and run at 2°C and pH8.5 at a constant current of 2.5mA per gel as described by Barton (1972). (2) Purified component C (1mg/ml) was treated with sodium dodecyl sulphate (1%, w/v), 2-mercaptoethanol (0.5%) and sucrose (6%, w/v) and then placed in a boiling-water bath for 5min. Samples of the resulting solution (10–80 μl) were then placed in wells in the top of a 10–30% (w/v) acrylamide exponential-gradient slab gel (20cm×17cm×0.15cm) containing 0.1% sodium dodecyl sulphate and run at room temperature (about 20°C) and pH8.5 at a constant current of 15mA. The apparatus and the methods used were similar to those of O'Farrell (1975). Gels were stained for protein with Coomassie Brilliant Blue R.

Colorimetric metal analysis

A sample of purified component C (4ml, 8mg of protein/ml) was dialysed for 24h against 1000vol. of 20mM-Tris/HCl buffer, pH7, containing 5mM-sodium thioglycollate and then divided into four portions. Two portions were assayed for copper and iron by using 2,9-dimethyl-1,10-phenanthroline (neocuproine) and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) respectively as described by Johnson (1964) except that the assays were scaled down to 1ml. The other two portions were each treated with 1% (w/v) sodium dodecyl sulphate and placed in a boiling-water bath for 10min. The samples were then assayed for copper and iron as described above. Sodium dodecyl sulphate at this concentration has no effect on the colorimetric analyses.

Preparation of prosthetic group

Purified component C (4mg of protein/ml) was dialysed for 24h at 2°C against 1000vol. of glass-distilled water. All further operations were done in the dark. The solution was heated for 10min in a boiling-water bath, cooled and then centrifuged at 5000g for 5min to remove the denatured protein. The yellow–green fluorescent supernatant was then spotted directly on silica-gel plates for t.l.c. or diluted appropriately in 20mM-sodium phosphate buffer, pH7, for spectrophotometry or spectrofluorimetry.

Spectrophotometry

Absorption spectra were measured by using a Pye–Unicam SP.1800 spectrophotometer connected to an AR25 linear recorder. Fluorescence excitation and emission spectra were measured with a Hitachi

Perkin-Elmer MPF-3 fluorescence spectrophotometer.

Thin-layer chromatography

T.l.c. was used to identify the prosthetic group. Glass plates (5cm × 20cm) precoated with a 0.25mm-thick layer of silica gel H₂₅₄ were developed with either 5% (w/v) Na₂HPO₄·12H₂O (solvent 1) or with a mixture of saturated (NH₄)₂SO₄ solution (40ml), 8% (w/v) sodium acetate (9ml) and propan-2-ol (2ml) (solvent 2). After drying the plates, the fluorescent spots were located under a bench u.v. lamp at 366nm.

Photoreduction of component C

A solution of component C (1ml, 3.6mg of protein) in 20mM-sodium phosphate, pH7, containing 20mM-EDTA was placed in a 1ml quartz cuvette fitted with a Suba-seal stopper and made anaerobic by flushing with N₂. The cuvette was then placed in an ice bath in front of a slide projector and illuminated with an intensity of 100000lx. Absorption spectra were recorded at intervals.

Determination of the molecular weight of component C

This was done by gel filtration as described by Andrews (1965), with catalase (mol.wt. 230000), yeast alcohol dehydrogenase (mol.wt. 150000), *E. coli* alkaline phosphatase (mol.wt. 80000), pig heart malate dehydrogenase (mol.wt. 66000) and myoglobin (mol.wt. 16800) as marker proteins. A 2ml sample containing marker enzymes [100units (μmol/min) each], myoglobin (5mg) and purified component C (5mg of protein) was placed on a 90cm × 3.8cm column of Sephadex G-200 equilibrated with 20mM-Tris/HCl buffer, pH7, containing 5mM-sodium thioglycollate. The column was eluted with the same buffer and 2ml fractions were collected. The position of the marker proteins in the eluate was determined by the methods of Andrews (1965). The position of component C was determined by measuring its methane mono-oxygenase activity in the presence of fraction A (4.1mg) and fraction B (1.2mg) as described above.

Determination of the polypeptide molecular weight of component C

This was done by treating purified component C (2mg of protein/ml) with 1% (w/v) sodium dodecyl sulphate and 0.5% 2-mercaptoethanol at 100°C for 5min followed by polyacrylamide-gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate (Weber & Osborn, 1969). The marker proteins were bovine plasma albumin (mol.wt. 68000), catalase (polypeptide mol.wt. 60000), ovalbumin (mol.wt. 43000), yeast alcohol dehydrogenase (polypeptide mol.wt. 37500) and α-chymotrypsinogen A (mol.wt. 23700).

Results

Resolution of soluble extracts into three fractions

When methane mono-oxygenase activity is measured as a function of the amount of soluble extract present in the reaction mixture, a non-linear relationship is obtained, suggesting that the enzyme is multi-component (Colby & Dalton, 1976). DEAE-cellulose chromatography as described in the Materials and Methods section resolves the soluble extract into three fractions A, B and C. Fractions A and C are absolutely required for enzyme activity, whereas fraction B stimulates activity 2–4-fold depending on the preparation used (Table 1). The possibility that fraction B might stimulate activity because it contains a small amount of either fraction A or fraction C was excluded by incubating larger amounts of fraction B with either fraction A or C alone (see Table 1); in neither case was activity observed.

Fig. 1 shows the effect of varying the concentration of fraction C in the presence of constant amounts of fractions A and B. A linear relationship was obtained over the range 0–0.2mg of protein/ml, enabling a specific activity for fraction C to be calculated. Similarly shaped curves were obtained no matter what the stage of purity of the component C, so that comparable specific activities of component C could be calculated at all stages during its purification provided that the amounts of fractions A and B were kept constant.

Stability of fractions A, B and C

Fractions A and B retained complete activity on storage at 0°C for 24h and fraction A was stable when heated at 55°C for 10min. In contrast fraction C was very unstable, losing 60–90% of its activity on storage at 0°C for 20h. A variety of compounds were tested for their ability to stabilize fraction C (Table 2), but

Table 1. *Reconstitution of methane mono-oxygenase activity from fractions A, B and C*

Fractions A, B and C were prepared from crude soluble extract by DEAE-cellulose chromatography. Methane mono-oxygenase activity was determined by measuring epoxyethane formation from ethene over a 10 min period and reactions were initiated by adding the various fractions in the amounts indicated.

Additions (mg of protein)	Epoxyethane formed (μmol/min)
A (3.4); B (1.3); C (0.3)	0.28
A (3.4); B (1.3)	0
A (3.4); B (3.9)	0
A (3.4); C (0.3)	0.07
B (1.3); C (0.3)	0
B (3.9); C (0.3)	0

only sodium thioglycollate (1–10mM), dithiothreitol (5–10mM) and NADH (5mM) were effective. Sodium thioglycollate (5mM) was used to maintain the activity of component C during its purification.

Purification of component C

Component C was purified some 80-fold by the procedure described in the Materials and Methods section and the results of a typical purification run are given in Table 3. Throughout the purification methane mono-oxygenase activities were determined by measuring the rate of oxidation of ethene to

epoxyethane (see the Materials and Methods section). Nevertheless, purified component C retained its ability to support the oxidation of methane to methanol in the presence of fractions A and B.

Non-denaturing polyacrylamide-gel electrophoresis indicated that the purified component C contained a single protein species, whereas electrophoresis on exponential-gradient gels in the presence of sodium dodecyl sulphate showed a faint contaminating band running ahead of the major component.

Fig. 1. Variation of reconstituted methane mono-oxygenase activity with the concentration of fraction C

Methane mono-oxygenase activity was measured as described in the Materials and Methods section by using constant amounts of fractions A (3.4 mg of protein) and B (1.3 mg of protein) and increasing amounts of fraction C (0–0.63 mg of protein).

Table 2. Effect of various compounds on the stability of fraction C at 0°C

Fraction C was prepared from crude soluble extract by DEAE-cellulose chromatography and assayed for methane mono-oxygenase activity in the presence of fraction A (3.4 mg) and fraction B (1.3 mg). Samples of fraction C (0.5 ml) were kept at 0°C in small polythene tubes in the presence of the test compounds at the concentrations indicated. Activities were retested after 22 h. The following compounds had no stabilizing effect: ethene (50%, v/v, in the gas phase); methane (50%, v/v); glycerol (30%, v/v); EDTA (1 mM); FAD (0.1 mM); FMN (0.1 mM); sodium ascorbate (1 mM); bovine plasma albumin (20 mg/ml); spermine (5 mM); MgCl₂ (5 mM); FeSO₄ (0.1 mM); CuSO₄ (0.1 mM).

Test compound	Concn. (mM)	Activity (% of original)
None	—	5
Sodium thioglycollate	1	79
Sodium thioglycollate	5	112
Sodium thioglycollate	10	76
Reduced glutathione	1 or 5	0
L-Cysteine hydrochloride	1 or 5	5
2-Mercaptoethanol	1 or 5	0
Dithiothreitol	1	0
Dithiothreitol	5	115
Dithiothreitol	10	111
NADH	1	0
NADH	5	94

Table 3. Purification of component C from crude soluble extracts of *M. capsulatus* (Bath)

The methane mono-oxygenase activity of component C was determined in the presence of fixed amounts of fraction A (4.1 mg) and fraction B (1.2 mg). At each stage in the purification, activities were measured with a variety of component C concentrations. These activities were then plotted against component C concentration and a specific activity was calculated from the linear part of the curve (see Fig. 1 and the text). The enzyme activity units are measured as $\mu\text{mol/min}$.

Stage	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification factor
Crude soluble extract	128	10240	726	0.07*	100	1
DEAE-cellulose eluate 1 (fraction C)	66	429	577	1.34	79	19
(NH ₄) ₂ SO ₄ fraction	8	227	414	1.82	57	26
Sephadex G-75 eluate	63	113	302	2.70	42	38
DEAE-cellulose eluate 2	70	49	183	3.74	25	53
Sephadex G-200 eluate	37	15	85	5.71	12	81

* With 3 mg of crude soluble extract in the reaction mixture.

Molecular weight of component C

The molecular weight of the purified component C was determined by gel filtration as described in the Materials and Methods section and a value of 44 600 was obtained. The polypeptide molecular weight of component C, determined as described in the Materials and Methods section, was 39 000, indicating that component C probably consists of a single polypeptide chain. In all calculations of absorption coefficients etc., the molecular weight of component C has been taken to be $42\,000 \pm 3\,000$.

Spectral properties of component C

Solutions of component C are not visibly fluorescent when irradiated with 366 nm light. They are yellow, with absorption maxima at 270 and 465 nm and a shoulder at 395 nm (Fig. 2). Heating a solution of component C on a boiling-water bath for 10 min followed by centrifugation (see the Materials and Methods section) results in a yellow-green fluorescent supernatant and a white precipitate. The absorption spectrum of the supernatant is almost identical with that of authentic FAD (Fig. 2) with maxima at 260, 375 and 445 nm. Identification of the prosthetic group as FAD was done by t.l.c. The yellow fluorescent material was spotted on silica-gel plates and developed in two different solvent systems. Its

Fig. 2. Absorption spectra of purified component C and of its prosthetic group

Component C was purified and prosthetic group prepared from it as described in the Materials and Methods section. Absorption spectra were measured in 20 mM-sodium phosphate buffer, pH 7. (1), Purified component C (3.6 mg of protein/ml); (2), purified component C (1.4 mg of protein/ml); (3), prosthetic group (prepared by boiling a solution of component C containing 3.6 mg of protein/ml); (4), FAD (0.045 mM).

Fig. 3. Fluorescence emission and excitation spectra of the prosthetic group

Prosthetic group was prepared from a solution of purified component C (8 mg of protein/ml) as described in the Materials and Methods section. —, Emission spectrum with excitation at 450 nm; ---, excitation spectrum with emission at 520 nm.

R_F value in solvent 1 (see the Materials and Methods section) was 0.570, compared with 0.570 for authentic FAD, 0.425 for FMN and 0.336 for riboflavin. Its R_F value in solvent 2 was 0.208, compared with 0.208 for FAD and 0.124 for FMN. The fluorescence emission and excitation spectra of the prosthetic group (Fig. 3) were also identical with those expected of FAD, with a single emission peak at 518 nm and excitation maxima at 370 and 450 nm.

FAD: protein ratio

Assuming the molar absorption coefficient for FAD to be $11.3 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Whitby, 1953) and the molecular weight of component C to be 42 000, FAD: protein ratios of 0.85:1 and 1:1 were calculated from the A_{445} of the prosthetic group and from the A_{465} of component C respectively.

Reduction of component C by sodium dithionite, by NADH or by photoreduction

Treatment of purified component C with excess sodium dithionite results in the disappearance of the 465 nm maximum. Similarly, treatment with increasing concentrations of NADH anaerobically results in a progressive decrease in the size of the 465 nm maximum until the fully reduced spectrum is observed after the addition of 1.2 mol of NADH/mol of enzyme (Fig. 4), suggesting that component C accepts two electrons per molecule. Solutions of component C, like many other flavoproteins (Müller *et al.*, 1972), are also reduced by anaerobic photo-

Fig. 4. Anaerobic titration of purified component C with NADH

A cuvette containing purified component C (1 ml, 5.5 mg of protein) was fitted with a Suba-seal stopper and made anaerobic by flushing with N_2 for 15 min. NADH (3.5 mM) was added through the stopper and the absorption spectrum measured after each addition. Spectra have not been corrected for the change in volume. (1) No addition; (2) +10 μ l of NADH; (3) +30 μ l of NADH; (4) +40 μ l of NADH; (5) +60 μ l of NADH. The spectrum returned to curve (1) within 2 min of re-admitting air to the cuvette.

reduction in the presence of 20 mM-EDTA (Fig. 5), fully reduced enzyme being obtained after 28 min illumination at 100 000 lx. Solutions fully reduced by treatment with stoichiometric amounts of NADH or by photoreduction are rapidly re-oxidized by air (Figs. 4 and 5), the fully oxidized spectrum being observed 2 min after re-admission of air to the cuvette. A new spectral species absorbing in the 570–630 nm region was observed during anaerobic titration with NADH (Fig. 4) and also on partial photoreduction (Fig. 7); this probably indicates the formation of a neutral flavin semiquinone (Müller *et al.*, 1972).

Metal content of component C

Dialysed samples of component C were assayed for copper and iron before and after disruption with sodium dodecyl sulphate as described in the Materials and Methods section. The maximum copper content

detected was 0.04 atom per molecule of protein, whereas the amount of iron detected in different preparations varied from 1.3 to 1.5 atoms per molecule. This suggests that component C probably contains 2 atoms of iron per molecule of protein. No iron was detected colorimetrically if the sample of component C was not disrupted by boiling with sodium dodecyl sulphate before the determination. Boiling the sample without sodium dodecyl sulphate allowed colour development to occur, but on extraction with chloroform the colour remained largely associated with the denatured protein precipitate. These observations suggest that the iron is very tightly bound in the native protein in such a way that it is not available to the bathophenanthroline reagent. Assuming that the iron is necessary for enzyme activity, this tight binding may account for the lack of inhibition of methane mono-oxygenase activity observed with most chelating agents (Colby & Dalton, 1976; Stirling & Dalton, 1977).

Fig. 5. Anaerobic photoreduction of purified component C in the presence of EDTA

Experimental details are given in the Materials and Methods section. (1) Spectrum before illumination; (2) after 3 min illumination; (3) after 10 min illumination; (4) after 20 min illumination; (5) after 28 min illumination. The spectrum returned to curve (1) within 2 min of re-admitting air to the cuvette.

Discussion

The function of each of the three components of the methane mono-oxygenase complex is not yet clear. However, the fact that some enzyme activity is observed in the absence of component B suggests that the latter does not have an essential catalytic function; possibly it may affect the interaction between the essential components A and B. At present it seems most likely that component C is the protein responsible for electron transport from NADH to the hydroxylase, and indeed it has similar properties to the flavoprotein electron-transport components of other multicomponent hydroxylase systems, e.g. adrenodoxin reductase (Chu & Kimura, 1973), putidaredoxin reductase (Tsai *et al.*, 1971), NADH-rubredoxin reductase (Ueda & Coon, 1972) and NADH-cytochrome b_5 reductase (Iyanagi, 1977). All these proteins contain 1 molecule of FAD per molecule and have mol.wts. between 38000 and 55000. Unlike them, component C contains iron in addition to FAD, although a functional role for the metal has not yet been established. All these electron-transport components serve as a link between a two-electron donor (NADH or NADPH) and a one-electron acceptor such as adrenodoxin, putidaredoxin or a cytochrome, so that a half-reduced form of the flavoprotein might be expected to be an intermediate. The increase in absorbance at 570–630 nm observed on partial reduction of component C, suggesting the formation

of a neutral semiquinone (Müller *et al.*, 1972), might represent such a semi-reduced intermediate.

Evidence from substrate-, inhibitor- and electron-donor-specificity studies (Colby & Dalton, 1976; Colby *et al.*, 1977; Stirling & Dalton, 1977) has indicated that the methane mono-oxygenase of *M. capsulatus* (Bath) differs significantly from the methane mono-oxygenase of *Methylosinus trichosporium*. The latter comprises three proteins: (1) a CO-binding cytochrome *c* of mol.wt. 13000, (2) a copper protein of mol.wt. 47000 and (3) a small protein of mol.wt. 9400 (Tonge *et al.*, 1977). The purification and characterization of component C, a metalloflavoprotein, confirms the fundamental differences between these two enzyme systems that catalyse the same reaction in different micro-organisms.

We thank Mr. P. P. Taylor for excellent technical assistance. The work was funded through a Science Research Council Research Grant to H. D.

References

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Barton, R. J. (1972) *Biochem. J.* **129**, 983–985
- Chu, J.-W. & Kimura, T. (1973) *J. Biol. Chem.* **248**, 2089–2094
- Colby, J. & Dalton, H. (1976) *Biochem. J.* **157**, 495–497

- Colby, J., Stirling, D. I. & Dalton, H. (1977) *Biochem. J.* **165**, 395–402
- Dalton, H. (1977) *Arch. Microbiol.* **114**, 273–279
- Iyanagi, T. (1977) *Biochemistry* **16**, 2725–2730
- Johnson, W. C. (ed.) (1964) *Organic Reagents for Metals and Certain Radicals*, vol. 2, pp. 58–74, Hopkin and Williams, Chadwell Heath
- Kennedy, S. I. T. & Fewson, C. A. (1968) *Biochem. J.* **107**, 497–506
- Müller, F., Brüstlein, M., Hemmerich, P., Massey, V. & Walker, W. H. (1972) *Eur. J. Biochem.* **25**, 573–580
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Stirling, D. I. & Dalton, H. (1977) *Arch. Microbiol.* **114**, 71–76
- Tonge, G. M., Harrison, D. E. F. & Higgins, I. J. (1977) *Biochem. J.* **161**, 333–344
- Tsai, R. L., Gunsalus, I. C. & Dus, K. (1971) *Biochem. Biophys. Res. Commun.* **45**, 1300–1306
- Ueda, T. & Coon, M. J. (1972) *J. Biol. Chem.* **247**, 5010–5016
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Whitby, L. G. (1953) *Biochem. J.* **54**, 432–442