The Respiratory Chain of *Hydrogenomonas* H16

By MICHAEL C. BEATRICE* and J. BRIAN CHAPPELL

Department of Biochemistry, University of Bristol
Medical School, Bristol BS8 1TD, U.K.

(Received 12 June 1978)

The respiratory chain of *Hydrogenomonas* H16 is shown to consist of two pathways, one of which has as its oxidase and only cytochrome a high-potential *b*-type cytochrome which is concluded to be cytochrome *o*. The K_m values for the oxygen of the two pathways are consistent with the high-potential *b*-type cytochrome functioning as a relatively high-affinity oxidase.

Little is known about the electron-transport chain in *Hydrogenomonas*. Eight strains of hydrogen bacteria, including *Hydrogenomonas* H16, belonging to the genera *Hydrogenomonas*, *Norcardia* and coryneform bacteria, contain cytochromes *a*, *b*, *c* and *o* (Bernard *et al.*, 1974), and only in strain 11/X did autotrophic, as distinct from heterotrophic, growth result in a qualitative difference in cytochrome content. Bongers (1967) has reported that the predominant phosphorylative pathway for hydrogen oxidation in cell-free extracts of autotrophic cells of *Hydrogenomonas* H20 is the abbreviated electron-transport chain ($H_2 \rightarrow$ cytochrome *b* $\rightarrow O_2$). Whereas Bongers (1970*a,b*) concluded by growth studies that hydrogen oxidation in *Hydrogenomonas eutropha* is coupled to phosphorylation at two sites, Ishaque & Aleem (1970) have shown that three sites are involved in hydrogen oxidation.

It is shown that the respiratory chain of *Hydrogenomonas* H16 is branched, and the conflicting results from previous studies can be reconciled by interpreting them on the basis of branched pathways.

Materials and Methods

Media and growth of organism

Hydrogenomonas H16 (N.C.I.B. 10442) was grown at 32°C by batch culture in a liquid medium containing: 20 mM-potassium phosphate (pH 6.5), 16 mM-urea, 0.5 mM-MgSO₄ and 20 µM-ferrous ammonium sulphate (Bongers, 1967). For fructose-growing cells the medium, also containing 50 mM-fructose, was aerated after the air was passed through 20% (w/v) NaOH solution to remove CO₂ and through water to remove traces of NaOH. Like *Hydrogenomonas* H20 (Bongers, 1967), trace elements were unnecessary, since contaminating salts in the major constituents were sufficient for growth. The gas phase for auto-

trophic growth was H₂/O₂/CO₂ (7:2:1, by vol.). Since the growth of *Hydrogenomonas facilis* (Cohen & Burris, 1955) and *H. eutropha* (Repaske, 1962) was diminished when gas consumption resulted in a partial vacuum, a water-displacing device which maintained the gas mixture under atmospheric pressure was used (Repaske, 1962). A 10% inoculum by volume of autotrophic cells from a liquid batch culture in the stationary phase was added to the medium. The cells were harvested after 22–24 h of growth by centrifugation at 6000g for 15 min at 10°C in the 6 × 250 ml head of an MSE 18 centrifuge.

Membrane preparation

The cells were washed twice by resuspension in a solution of 150 mM-KCl/15 mM-Tris/HCl, pH 7.4, and re-centrifuging at 6000g for 15 min at 10°C in the 8 × 50 ml head of an MSE 18 centrifuge. The washed cells were suspended in breaking medium, which was 150 mM-KCl/15 mM-Tes (2-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino}ethanesulphonic acid), pH 7.0. An equal volume of glass beads (100 mesh; BDH Chemicals, Poole, Dorset, U.K.) and a few grains of crude deoxyribonuclease were then added. This slurry was shaken for 2.5 min at the higher speed of a Braun MSK mechanical cell homogenizer (Shandon Scientific Co., Willesden, London N.W. 10, U.K.). After filtration through cotton muslin to remove the beads, the preparation was centrifuged at 6000g for 15 min at 10°C in the 8 × 50 ml head of an MSE 18 centrifuge. The 6000g supernatant was centrifuged at 38000g at 10°C for 30 min in the 8 × 50 ml head of an MSE 18 centrifuge. The 38000g precipitate was suspended in the experimental medium to a protein concentration of approx. 40 mg/ml.

Continuous measurement of oxygen partial pressure

Oxygen partial pressure was measured as described by Chappell (1964) and Estabrook (1967). When hydrogen was substrate, the oxygen electrode was first

* Present address: Hormel Institute, University of Minnesota, 801 16th Avenue N.E., Austin, MN 55912, U.S.A.

calibrated with air-saturated medium. Hydrogen-saturated medium, 1 μ l of catalase diluted 5-fold, and sufficient H_2O_2 to give an O_2 concentration equal to that in air-saturated medium were added. The membrane fraction was added last to initiate the reaction.

Potentiometric titration of cytochromes

The technique for the determination of the redox potential of membrane-bound cytochromes has been described (Dutton, 1971; Dutton *et al.*, 1970; Wilson & Dutton, 1970*a,b*). Absorbance changes were followed in a dual-wavelength spectrophotometer and the oxidation-reduction potential was measured by using a platinum electrode and a standard calomel electrode. Also 0.25 μ l of antifoam emulsion/mg of protein was added for the prevention of foam generated by a N_2 stream which maintained anaerobiosis.

The oxidation-reduction chemicals were [with mid-point oxidation-reduction potential at pH 7 (E'_0) in parentheses]: (A) potassium ferri-ferrocyanide ($E'_0 = +430\text{mV}$); (B) diaminoduroil ($E'_0 = +240\text{mV}$); (C) phenazine methosulphate ($E'_0 = +80\text{mV}$); (D) phenazine ethosulphate ($E'_0 = +55\text{mV}$). Sufficient potassium ferricyanide was added to attain a potential of +490mV. The oxidation-reduction potential of the system was slowly diminished by endogenous substrate and by successive additions of small quantities of NADH. The three mediator dyes were added at the start of the experiment, since no difference occurred by adding them at the appropriate electrode potential of the oxidation-reduction system.

Assays

The protein content of the membrane preparation was assayed by the method of Gornall *et al.* (1949). Cytochrome *o* was determined from the dithionite-reduced + CO-minus-dithionite-reduced difference spectrum by using $\epsilon_{\text{mM}} = 85$ for the peak plateau 417–460nm (Chance, 1961). Other cytochromes were determined by using the absorption coefficients proposed by Chance & Williams (1956) for the mitochondrial cytochromes.

Reagents

NADH (disodium salt) was purchased from Boehringer Corp., London W.5, U.K., and Silicone MS Antifoam Emulsion FG from Hopkin and Williams, Chadwell Heath, Essex, U.K. Bovine serum albumin (fraction V), used as a standard in the protein determination, and the deoxyribonuclease (EC 3.1.4.5, product no. DN-25) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Catalase (EC 1.11.1.6) was purchased from BDH. The mediator dyes were gifts from Dr. A. R. Crofts (Department of Biochemistry, University of Bristol Medical School). All other reagents were from BDH.

Results and Discussion

Inhibition of O_2 uptake by KCN or CO

The particulate fraction from autotrophic cells (2mg of protein/ml) oxidized hydrogen, NADH (1.3mM), succinate (6.25mM) and ascorbate (5mM) in the presence of *NNN'*-tetramethyl-*p*-phenylenediamine (0.1mM). The ascorbate oxidase did not proceed without tetramethylphenylenediamine. KCN (2mM) caused a 39 and 98% inhibition of the rate of the NADH oxidase and ascorbate-tetramethylphenylenediamine oxidase respectively, but had no effect on the low rate of succinate oxidase, which was less than the inhibited rates of the oxidases.

Fig. 1 shows that 1mM-KCN inhibited hydrogen oxidation by approx. 40% and ascorbate-tetramethylphenylenediamine oxidation by 91 and 99% after 5.5min. CO inhibited the hydrogen oxidase, NADH oxidase and ascorbate-tetramethylphenylenediamine oxidase by over 85%.

Cytochrome spectra of autotrophically grown cells

Dithionite, hydrogen, NADH and succinate

Fig. 1. Oxygen-electrode traces of hydrogen and ascorbate-tetramethylphenylenediamine oxidation

For hydrogen oxidation (traces *a* and *b*) catalase and H_2O_2 were added to hydrogen-saturated solution to give an O_2 concentration equal to that in the air-saturated medium (traces *c* and *d*). The membrane fraction (F) was added last to initiate the reaction. The membrane fraction was suspended at 0.45mg of protein/ml in 150mM-KCl/15mM-Tes at 25°C and pH 7.0. The additions were: sodium ascorbate (1.25mM; Asc), tetramethylphenylenediamine (25 μ M; TMPD) and KCN (1mM).

reduce *b*-, *c*- and *a*-type cytochromes. The NADH- and succinate-reduced difference spectra developed slowly because of the low rate of O₂ consumption by particles utilizing NADH or succinate. When dithionite was added to the test cuvettes of the substrate-reduced-minus-oxidized spectra, further reduction occurred, but primarily of the *b*-type cytochrome. The ascorbate-tetramethylphenylenediamine-reduced-minus-oxidized difference spectrum in Fig. 2(a) shows that ascorbate reduces *c*- and *a*-type cytochromes. Cytochrome *c* had α -, β - and γ -bands of 553, 523 and 422–423 nm respectively; cytochrome *a*

Fig. 2. Cytochrome spectra

(a) 4 mM-Sodium ascorbate and 20 μ M-tetramethylphenylenediamine were added to the test cuvette. The medium was 150 mM-KCl/15 mM-Tes, pH 7.0 at 25°C. The bottom trace was obtained by adding only the membrane fraction to both cuvettes. (b) Dithionite was added to the test cuvette: 4 mM-sodium ascorbate and 50 μ M-tetramethylphenylenediamine were added to the reference cuvette. Other conditions were as in (a).

Fig. 3. Dithionite-reduced + CO-minus-dithionite-reduced difference spectrum

Dithionite was in both cuvettes, and the test cuvette was flushed with CO for 15 min. The bottom trace was obtained by adding dithionite to both cuvettes. Other conditions were as in Fig. 2(a).

had α - and γ -bands of 600 and 442–444 nm respectively. The dithionite-reduced-minus-ascorbate-tetramethylphenylenediamine-reduced difference spectrum in Fig. 2(b) shows a *b*-type cytochrome which has α -, β - and γ -bands of 562, 532 and 432 nm respectively. The dithionite-reduced + CO-minus-dithionite-reduced difference spectrum (Fig. 3) demonstrates the existence of cytochrome *o*. The spectrum of the cytochrome *o*-CO complex has a trough at 562 nm and the α -, β - and γ -bands at 574, 543 and 419 nm respectively. Full formation of the complex with CO required 15 min flushing with CO.

An experiment that was conducted with cell-free extracts of autotrophic cells of *Hydrogenomonas* H20 (Bongers, 1967) is shown in Fig. 4(a). Cytochrome 562, which was reduced along with cytochrome 553 and cytochrome 600 on flushing the test cuvette with hydrogen (Fig. 4a, curve A), was oxidized on flushing the test cuvette with O₂ in the presence of KCN. Cytochrome 553 and cytochrome 600 remained to a great degree reduced (Fig. 4a, curve B). The flushing of the test cuvette with O₂ in the absence of KCN resulted in the oxidation of all cytochromes (Fig. 4a, curve C). The addition of KCN alone resulted in a small partial reduction of cytochrome 553 and cytochrome 600 due to the presence of endogenous sub-

Fig. 4. Effect of KCN on cytochromes reduced by hydrogen and NADH

(a) A, Hydrogen was flushed into the test cuvette for 3 min. B, The spectrum obtained from A when O_2 was flushed into the test cuvette for 30 s after the addition of 1.0 mM-KCN to the test cuvette. C, The spectrum obtained from A when O_2 was flushed into the test cuvette for 15 s in the absence of cyanide. Other conditions were as in Fig. 2(a). (b) A, 1 mM-NADH was added to the test cuvette, and 4 min elapsed before the trace was fully developed. B, The spectrum obtained from A when O_2 was flushed into the test cuvette for 5 s after the addition of 1.0 mM-KCN to the test cuvette. C, The spectrum obtained from A when O_2 was flushed into the test cuvette for 5 s in the absence of cyanide. Other conditions were as in Fig. 2(a).

strate. Similar results were obtained when NADH replaced hydrogen as the reductant (Fig. 4b). NADH added to a test cuvette containing KCN instantaneously reduced cytochrome 553 and cytochrome 600 (Fig. 5a, curve A). Cytochrome 562 was subsequently reduced and there was a further reduction of cytochrome 553 of about 14% (Fig. 5a, curve B). Cytochrome 562 was reduced by a further approx. 50% when the test cuvette was flushed with hydrogen (Fig. 5a, curve C). Fig. 5(b) shows the effect of CO on the cytochromes. The medium in which the particulate fraction was suspended was deoxygenated by O_2 -free N_2 . Succinate added to a test cuvette containing KCN resulted in the virtually instantaneous reduction of cytochrome 553 and cytochrome 600 (Fig. 5b, curve A). When the test cuvette was flushed with CO, cytochrome 562 appeared (Fig. 5b, curve B), and was reoxidized when the test cuvette was flushed with O_2 . Dithionite, added after the CO, reduced cytochrome 562 by a further 40% (Fig. 5b, curve C). Similar results were obtained when NADH replaced succinate as reductant. When the test cuvette was flushed with CO in the absence of KCN and substrate all cytochromes were reduced.

Potentiometric titration of cytochromes

The potentiometric titration of cytochrome 553 indicates two components, which are two-electron acceptors and have E'_0 values of +264 mV and +150 mV (Fig. 6). Cytochrome 562 consisted of a one-electron acceptor with an E'_0 of +96 mV (Fig. 6) and a two-electron acceptor with an E'_0 of +418 mV (Fig. 7). Cytochrome 600 was a one-electron acceptor with an E'_0 value of +307 mV (Fig. 7).

Nature of the respiratory chain

That ascorbate-tetramethylphenylenediamine reduced cytochrome 553 and cytochrome 600 to at least the same degree as NADH, hydrogen or succinate without reducing cytochrome 562, whereas the organic substrates reduced cytochrome 562, implies that cytochrome 562 does not follow either cytochrome 553 or 600. Since reduced cytochrome 562, unlike the other two cytochromes, reacts with O_2 in the presence of KCN, the ascorbate-tetramethylphenylenediamine oxidase would not be completely inhibited by KCN in an air-saturated medium if cytochrome 562 followed either of the other two cytochromes. Although KCN inhibited the ascorbate-

Fig. 5. Effect of KCN and CO on the reduction of cytochromes

(a) A, 1 mM-NADH was added to the test cuvette after the addition of 1 mM-KCN, and the spectrum recorded immediately. B, The spectrum obtained from A after 15 min. C, The spectrum obtained from B after flushing the test cuvette with hydrogen for 3 min. Other conditions were as in Fig. 2(a). (b) A, 15 mM-sodium succinate was added to the test cuvette after the addition of 1 mM-KCN and the spectrum recorded immediately. B, The spectrum obtained from A after flushing the test cuvette with CO for 30 s. C, The spectrum obtained when dithionite was added to the test cuvette of B. Other conditions were as in Fig. 2(a).

Fig. 6. Oxidation-reduction titration of cytochrome 553 and the low-potential component of cytochrome 562

(a) The membrane fraction (8.9 mg of protein/ml) was suspended in 140 mM-KCl and 20 mM-Tes at pH 7.0 and 25°C. The wavelength pair was 553–540 nm. Potassium ferricyanide was added to oxidize the system. Diaminodureol (65 μM), phenazine methosulphate (35 μM) and phenazine ethosulphate (35 μM) were then added, and the titration was conducted.

tetramethylphenylenediamine oxidase, oxidation of NADH and hydrogen was only slightly diminished. Hence there exists a respiratory pathway that includes cytochrome 553 and cytochrome 600 and is cyanide- and CO-sensitive, and an alternative cyanide-insensitive CO-sensitive pathway that does not include the *c*- and *a*-type cytochromes.

To test whether one pathway had a greater affinity for oxygen than the other, the K_m values for O_2 of the separate pathways were determined by the 'critical O_2 ' concentration (Meyer & Jones, 1973). Particulate fractions from both autotrophic and fructose-grown cells were used. The cyanide-sensitive pathway was assayed by using ascorbate-tetramethylphenylenediamine, and the cyanide-resistant pathway by using

(b) The membrane fraction was 6.1 mg of protein/ml and the wavelength pair was 562–540 nm for titration of cytochrome 562. Other conditions were as in Fig. 6(a).

hydrogen or NADH in the presence of 0.2 mM-KCN, and both pathways were assayed in its absence. KCN (0.2 mM) inhibited the ascorbate-tetramethylphenylenediamine oxidase of both autotrophic and fructose-grown cells by over 93%. The average K_m of the cyanide-resistant pathway for both sets of cells was approx. $3.0 \mu\text{M}$; the average K_m values of the cyanide-sensitive pathway of membrane fragments from autotrophic and fructose-grown cells were 9.8 and $14.5 \mu\text{M}$ respectively (Table 1). The K_m value when both pathways were operative was that of the pathway with the greater affinity for O_2 (the cyanide-resistant pathway). This K_m of a pathway in which cytochrome o (b_H) is the oxidase is comparable with the K_m of other bacterial pathways in which cytochrome o is the sole or major oxidase (Meyer & Jones, 1973).

Fig. 7. Oxidation-reduction titration of cytochrome 600 and the high-potential component of cytochrome 562 (a) The membrane fraction was 6.1 mg of protein/ml and the wavelength pair was 562–540 nm for titration of cytochrome 562. Other conditions were as in Fig. 6(a). (b) The membrane fraction was 10.0 mg of protein/ml and the wavelength pair was 600–574 nm. Other conditions were as in Fig. 6(a).

Cyanide inhibition of the ascorbate-tetramethylphenylenediamine oxidase has been examined by following O_2 uptake in the presence of different KCN concentrations. A semi-logarithmic plot of the rate of O_2 uptake versus time yielded parallel lines. Since the slopes were not proportional to cyanide concentration, inhibition of O_2 uptake with time, unlike mitochondrial cytochrome oxidase (Nichols *et al.*, 1972; Yoshikawa & Orii, 1972) and the cytochrome o of *Escherichia coli* (Pudek & Bragg, 1974), cannot be explained by assuming that the rate of reaction of the inhibitor with the oxidase to form an enzyme-inhibitor complex was rate-limiting.

If both components of cytochrome 562 have similar absorption coefficients, the molar ratio of the high-potential (b_H) to low-potential component (b_L) can be estimated to be 5.1:1. The reduction of cytochrome 562 by CO under conditions when all the other cytochromes were fully reduced implies that cytochromes o and b_H are identical. Whereas according to the preceding ratio b_H was 85% of the total cytochrome b content, cytochrome o can be estimated to be 75% of the total. Cytochrome b_H with its high midpoint potential of +418 mV and CO-sensitivity is concluded to be cytochrome o and the oxidase of the relatively cyanide-insensitive CO-sensitive pathway.

The particulate fraction of fructose-grown cells collected in the early stationary phase had a cytochrome b_H/b_L ratio of 1:4.7; 17% of the total cytochrome b content was b_H . The flushing with O_2 of a test cuvette, containing 1 mM-KCN, of an NADH-reduced-minus-oxidized difference spectrum (Fig. 8, curve a), unlike those from autotrophically grown cells, did not result in the oxidation of cytochrome 562 (Fig. 8, curve b). Reduced cytochrome 562 declined by about 20%, which is the estimated proportion of cytochrome 562 that is b_H . Ascorbate-tetramethylphenylenediamine reduced not only cytochrome 553 and cytochrome 600, but, unlike autotrophic cells, cytochrome 562 as well. Since cytochrome 562 of autotrophic cells consisted predominantly of b_H , whereas cytochrome 562 of fructose-grown cells consisted

Table 1. K_m values of the two pathways for O_2 determined from membrane fractions from autotrophic and fructose-grown cells. Each value is given $\pm$ S.E.M. with the number of observations in parentheses. Membrane fractions from autotrophic and fructose-grown cells were suspended in 15 mM-Tris/HCl/150 mM-KCl medium at pH 7.4 and 25°C. Abbreviation: TMPD, tetramethylphenylenediamine.

	Pathway	Substrate	K_m for O_2 (μM)
Autotrophic cells	Cyanide-sensitive	2.1 mM-Ascorbate + 42 μM -TMPD	9.8 ± 0.2 (3)
	Cyanide-resistant	H_2 (+0.2 mM-KCN)	3.2 ± 0.2 (3)
		1.8 mM-NADH (+0.2 mM-KCN)	2.8 ± 0.3 (3)
		H_2	3.0 ± 0.2 (4)
	Both pathways	1.8 mM-NADH	2.7 ± 0.4 (3)
Fructose-grown cells	Cyanide-sensitive	2.1 mM-Ascorbate + 42 μM -TMPD	14.5 ± 1.0 (3)
	Cyanide-resistant	1.8 mM-NADH (+0.2 mM-KCN)	2.9 ± 0.4 (3)
	Both pathways	1.8 mM-NADH	2.6 ± 0.5 (3)

Fig. 8. Absorption spectra of the membrane fraction from fructose-grown cells
(a) The spectrum obtained when 1.6 mM-NADH was added to the test cuvette containing 1 mM-KCN. (b) The spectrum obtained from (a) after flushing the test cuvette with O₂ for 20 s. (c) The spectrum obtained when the test cuvette of the NADH-reduced-minus-oxidized difference spectrum was flushed with O₂ for 10 s in the absence of cyanide. Other conditions were as in Fig. 6(a).

predominantly of *b_L*, ascorbate-tetramethylphenylenediamine reduces only cytochrome *b_L*.
Studies of the effect of KCN on the rate of O₂ uptake by the particulate fraction from fructose-grown cells showed that the effect was similar to preparations from autotrophic cells, except that hydrogen was not oxidized. Thus 0.9 mM-KCN inhibited the ascorbate-tetramethylphenylenediamine oxidase by 92 %, and the subsequent addition of NADH stimulated O₂ uptake; 0.9 mM-KCN inhibited the NADH oxidase by 40 %. Since cyanide inhibits O₂ uptake by preparations utilizing ascorbate, which reduces cytochrome *b_L*, it is concluded that the cyanide-insensitive cytochrome *b_H* is on a branch from a point preceding cytochrome *b_L* as well as cytochrome 553 and cytochrome 600.
The electron-transport chain in Scheme 1 satisfies the experimental findings. It is similar to the respira-

Scheme 1. Scheme for the electron-transport chain of *Hydrogenomonas* H16

tory chain of free-living *Rhizobium japonicum* (Appleby, 1969). The branch point may be a quinone, since ubiquinone Q₈ occurs in *Hydrogenomonas eutropha* (Lester & Crane, 1959) and a quinone has been reported in *Hydrogenomonas* H20 (Bongers, 1967) and *Hydrogenomonas* H16 (Jones *et al.*, 1975). Another possibility is of two distinct chains: a short chain consisting of only one cytochrome, *b_H*, and a long one consisting of all the other cytochromes.

Schlegel & Lafferty (1971) have demonstrated that, when the O₂-supply rate satisfies only half the O₂-consumption rate of autotrophically growing *Hydrogenomonas* H16, normal exponential growth is maintained. Bongers (1970*a,b*) by growth studies on *H. eutropha* concluded that hydrogen oxidation is coupled at two phosphorylation sites. Ishaque & Aleem (1970), however, have shown that there are three sites. These findings can be explained by interpreting them on the basis of a branched respiratory chain.

References

- Appleby, C. A. (1969) *Biochim. Biophys. Acta* **172**, 88–105
 Bernard, U., Probst, I. & Schlegel, H. G. (1974) *Arch. Mikrobiol.* **95**, 29–37
 Bongers, L. (1967) *J. Bacteriol.* **93**, 1615–1623
 Bongers, L. (1970*a*) *J. Bacteriol.* **104**, 145–151
 Bongers, L. (1970*b*) *J. Bacteriol.* **102**, 598–599
 Chance, B. (1961) in *Haematin Enzymes* (Falk, J. E., Lemberg, R. & Morton, R. K., eds.), part 2, pp. 433–435, Pergamon Press, New York
 Chance, B. & Williams, G. R. (1956) *Adv. Enzymol. Relat. Subj. Mol. Biol.* **17**, 65–134
 Chappell, J. B. (1964) *Biochem. J.* **90**, 225–237
 Cohen, J. S. & Burris, R. H. (1955) *J. Bacteriol.* **69**, 316–319
 Dutton, P. L. (1971) *Biochim. Biophys. Acta* **226**, 63–80
 Dutton, P. L., Wilson, D. F. & Lee, C.-P. (1970) *Biochemistry* **9**, 5077–5082
 Estabrook, R. W. (1967) *Methods Enzymol.* **10**, 41–47
 Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
 Ishaque, M. & Aleem, M. I. H. (1970) *Biochim. Biophys. Acta* **223**, 388–397
 Jones, C. W., Brice, J. M., Downs, A. J. & Drozd, J. W. (1975) *Eur. J. Biochem.* **52**, 265–271
 Lester, R. L. & Crane, F. L. (1959) *J. Biol. Chem.* **234**, 2169–2175
 Meyer, D. J. & Jones, C. W. (1973) *FEBS Lett.* **33**, 101–105
 Nichols, P., Van Buuren, K. J. H. & Van Gelder, B. F. (1972) *Biochim. Biophys. Acta* **275**, 279–287
 Pudek, M. R. & Bragg, P. D. (1974) *Arch. Biochem. Biophys.* **164**, 682–693
 Repaske, R. (1962) *J. Bacteriol.* **83**, 418–422
 Schlegel, H. G. & Lafferty, R. M. (1971) *Adv. Biochem. Eng.* **1**, 143–168
 Wilson, D. F. & Dutton, P. L. (1970*a*) *Arch. Biochem. Biophys.* **136**, 583–584
 Wilson, D. F. & Dutton, P. L. (1970*b*) *Biochem. Biophys. Res. Commun.* **39**, 59–64
 Yoshikawa, S. & Orii, Y. (1972) *J. Biochem. (Tokyo)* **71**, 859–872