Isolation and Properties of a 'Malic' Enzyme from Cauliflower Bud Mitochondria

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1. A 'malic' enzyme [L-malate-NAD oxidoreductase (decarboxylating), EC 1.1.1.39] has been isolated from cauliflower bud mitochondria and partially purified. 2. The enzyme is specific for L-malate and has an absolute requirement for either Mn^{2+} , Co^{2+} or Mg^{2+} . 3. The enzyme shows activity with both NAD^+ and $NADP^+$, but NAD^+ is the preferred cofactor. 4. No appreciable oxaloacetate decarboxylase activity is present in the enzyme preparations even at low pH values. 5. The enzyme is inhibited by NADH and by oxaloacetate and stimulated by SO_4^{2-} and by low concentrations of CoA. 6. The regulatory properties of the enzyme support the proposed role of the enzyme in the utilization of tricarboxylic acid-cycle acids for energy production when glycolysis is suppressed.

Recent work has shown that pyruvate is the major product of malate oxidation by isolated cauliflower bud mitochondria when pyruvate dehydrogenase is inhibited (Macrae & Moorhouse, 1970). The pyruvate is formed directly from malate by a mitochondrial NAD-requiring 'malic' enzyme [L-malate-NAD oxidoreductase (decarboxylating), EC 1.1.1.39]. This pathway appears to be present in a number of plant mitochondrial preparations which show rapid malate oxidation rates (Macrae, 1971) and may also operate in *Ascaris* muscle mitochondria (Papa, Cheah, Rasmussen, Lee & Chance, 1970).

NAD-requiring 'malic' enzymes occur in some micro-organisms (Ochoa, Verga Salles & Ortiz, 1950; Nathan, 1961; Temperli, Kunsch, Meyer & Busch, 1965; London & Meyer, 1969*a*; Takeo, 1969) and in invertebrates (Saz & Hubbard, 1957; Pritchard & Schofield, 1968), but had not been detected in higher plants. Therefore, in this paper I report the partial purification and some properties of the 'malic' enzyme from cauliflower bud mitochondria. The properties of the enzyme are compared with those of other 'malic' enzymes. Because of the proposed role of the mitochondrial enzyme in the utilization of malate by plants (Macrae & Moorhouse, 1970) and the regulatory properties of some bacterial 'malic' enzymes (Sanwal & Smando, 1969; Sanwal, 1970; London & Meyer, 1969*b*), the influence of effectors of possible regulatory importance on the activity of the enzyme is described.

EXPERIMENTAL

Materials. Cauliflowers were purchased locally and stored for up to 1 week at 4°C before use. Malate dehydrogenase (EC 1.1.1.37) was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Other biochemicals were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Sephadex and DEAE-Sephadex were supplied by Pharmacia (G.B.) Ltd., London W.13, U.K.

Enzyme assays. 'Malic' enzyme and malate dehydrogenase were assayed by spectrophotometric measurement of oxidation or reduction of nicotinamide nucleotides with a Unicam SP800 spectrophotometer fitted with a scale-expansion unit and external recorder. Changes in E_{340} were measured against blanks containing substrates but no enzymes.

For 'malic' enzyme the standard incubation mixture contained, in a final volume of 3.0 ml, 15 μ mol of L-malate, 6 μ mol of $MnCl_2$, 3 μ mol of $NAD(P)^+$, 60 μ mol of TES* and enzyme. The final pH was 6.8 and the incubation temperature 25°C.

For malate dehydrogenase the standard incubation mixture contained, in a final volume of 3.0 ml, 3 μ mol of oxaloacetate, 1.5 μ mol of NADH, 60 μ mol of TES and enzyme. The final pH was 6.8 and the incubation temperature 25°C.

Assay of reaction products. Pyruvate and malate were assayed by using enzyme reactions coupled to oxidation or reduction of nicotinamide nucleotides (Macrae & Moorhouse, 1970).

Protein determination. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

* Abbreviation: TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

Isolation and purification of the 'malic' enzyme. All steps were carried out at 0–5°C.

Mitochondria were prepared from about 300 g of cauliflower buds as described by Macrae & Moorhouse (1970). The mitochondria were suspended in 10 ml of 20 mM-TES buffer, pH 6.8, containing 50 mM-mercaptoethanol and 5 mM-MgCl₂. The resulting mixture was sonicated for 3 min (power output about 50 W) and then centrifuged at 150 000 g for 30 min. The supernatant, which contained the crude 'malic' enzyme, was collected and a small sample was assayed for 'malic' enzyme, malate dehydrogenase and protein content.

After dilution of the remainder of the supernatant to 30 ml, (NH₄)₂SO₄ was added to it. The fraction precipitating between 0 and 60% saturation was collected and dissolved in a small quantity of the TES buffer. A portion was assayed for enzyme and protein content. After removal of (NH₄)₂SO₄ with a Sephadex G-25 column, the remainder of the solution was diluted to 20 ml with the TES buffer and a second (NH₄)₂SO₄ fractionation was performed. The fraction precipitating between 40 and 55% saturation was collected and dissolved in a small volume of TES buffer. A sample of this partially purified 'malic' enzyme solution was assayed for enzyme and protein content.

After removal of (NH₄)₂SO₄ with Sephadex G-25 the partially purified 'malic' enzyme solution was chromatographed on a DEAE-Sephadex A-25 column (25 cm × 1.5 cm) by elution with a linear gradient of 0–0.4 M-KCl in 20 mM-tris buffer, pH 7.2, containing 50 mM-mercaptoethanol and 5 mM-MgCl₂ (total volume 300 ml). The flow rate was 80 ml/h. Fractions (7.5 ml) were collected and assayed for 'malic' enzyme activity with both NAD⁺ and NADP⁺. A single peak of activity, eluted at 0.23 M-KCl, was obtained. The fractions containing the activity were concentrated to a small volume with an Amicon Diaflo Ultrafilter (PM-30). This purified enzyme solution was assayed for 'malic' enzyme, malate dehydrogenase and protein content.

RESULTS

Enzyme purification. Protein recoveries and the specific activities of the 'malic' enzyme at the various stages of purification are shown in Table 1. A 14-fold purification of the enzyme was achieved

by ammonium sulphate fractionation and DEAE-Sephadex chromatography. In its purest form the 'malic' enzyme showed activity with both NAD⁺ and NADP⁺, but had no appreciable malate dehydrogenase (L-malate-NAD oxidoreductase; EC 1.1.1.37) activity.

The poor recovery of 'malic' enzyme activity after DEAE-Sephadex chromatography can be attributed to the instability of the enzyme in solution. Dilute solutions of the enzyme were found to lose activity rapidly, especially at high salt concentrations. This loss of activity was slowed down by addition of 50 mM-mercaptoethanol, but other possible protectors tested were without effect. Because of the instability of the enzyme in solution, the partially purified enzyme obtained by ammonium sulphate fractionation was used in most of the experiments described below. The precipitated protein could be stored at –20°C with only slow loss of 'malic' enzyme activity.

Stoichiometry of the 'malic' enzyme reaction. Previous results showed that pyruvate formation by a crude 'malic' enzyme preparation was stoichiometric with NAD(P)⁺ reduction under a variety of conditions (Macrae & Moorhouse, 1970). This stoichiometry was confirmed by using the partially purified enzyme in a prolonged incubation from which samples were taken at 5 min intervals and assayed for NADH and pyruvate formation (Table 2).

pH optimum. The 'malic' enzyme reaction rate as a function of pH is shown in Fig. 1. With 5 mM-malate, 1 mM-NAD⁺ and 2 mM-Mn²⁺ the optimum pH for the reaction was between pH 6.7 and 6.9.

Substrate specificity. The partially purified enzyme was highly specific for L-malate. Citrate, isocitrate, fumarate, succinate and tartronate were totally ineffective as substrates for the enzyme, and D-malate and β-hydroxybutyrate reacted at approximately one-hundredth of the rate with L-malate. At pH 6.8, with 1 mM-NAD⁺ and 2 mM-Mn²⁺, the K_m for L-malate was 0.48 mM.

Table 1. *Purification of 'malic' enzyme*

Assay conditions were used as described in the Experimental section.

Fraction	Total protein (mg)	Specific activities [μmol of NAD(P)H/min per mg of protein]			Recovery of NAD 'malic' enzyme (%)
		NAD 'malic' enzyme	NADP 'malic' enzyme	Malate dehydrogenase	
Crude enzyme	11.4	0.33	0.07	14.9	—
1st (NH ₄) ₂ SO ₄ precipita- tion	2.6	0.98	0.14	2.29	67
2nd (NH ₄) ₂ SO ₄ precipita- tion	2.0	1.07	0.15	0.33	58
DEAE-Sephadex fraction	0.14	4.71	0.69	0.00	17

Table 2. *Stoichiometry of 'malic' enzyme reaction*

The reaction mixture contained 5mM-L-malate, 1mM-NAD⁺, 2mM-Mn²⁺, 20mM-TES and 240μg of partially purified enzyme in a total volume of 20ml. The final pH was 6.8 and the incubation temperature 25°C. Samples (3.0ml) were taken at 5min intervals and assayed for product formation.

Incubation time (min)	Concn. of NADH (mM)	Concn. of pyruvate (mM)
5	0.059	0.059
10	0.121	0.121
15	0.161	0.163
30	0.217	0.202
25	0.240	0.239
30	0.275	0.273

Fig. 1. Effect of pH on the 'malic' enzyme reaction. Except for the variation in pH, the standard 'malic' enzyme assay system was used with NAD⁺ as cofactor.

Coenzyme requirement. NAD⁺ was the most effective cofactor for the mitochondrial 'malic' enzyme. However, at all stages of the enzyme purification some activity was also obtained with NADP⁺ (Table 1). The retention of activity with both NAD⁺ and NADP⁺ after ammonium sulphate fractionation and DEAE-cellulose chromatography indicates that the cauliflower mitochondrial 'malic' enzyme has dual nicotinamide nucleotide specificity. The increase in the ratio of activity with NAD⁺ to activity with NADP⁺ from 5 to 7 as a result of the first ammonium sulphate fractionation suggests that the sonicated mitochondrial preparation was contaminated by a small amount of soluble NADP⁺-requiring 'malic' enzyme, which was removed during the initial stages of the purification procedure. At pH 6.8 with 5mM-malate and 2mM-Mn²⁺ the

Table 3. *K_m and V_{max} values for metal ions*

The reaction mixtures contained 15μmol of L-malate, 3μmol of NAD⁺, 60μmol of TES, various concentrations of metal ions (as chlorides) and 40μg of partially purified enzyme in a total volume of 3.0ml. Final pH was 6.8, and incubation temperature 25°C. V_{max} and K_m values were determined from plots of reaction rate against metal ion concentration.

Metal	V _{max} (μmol of NADH/min)	K _m (mM)
Mn ²⁺	0.028	0.02
Co ²⁺	0.018	0.06
Mg ²⁺	0.016	0.5

Table 4. *Reductive carboxylation of pyruvate*

The complete system contained 60μmol of pyruvate, 60μmol of HCO₃⁻, 6μmol of Mn²⁺, 1.5μmol of NADH, 60μmol of TES and 160μg of partially purified enzyme in a total volume of 3.0ml. Final pH was 6.8, and incubation time 1h at 25°C.

Incubation system	Malate formed (μmol)
Complete system	0.29
Enzyme omitted	0.01
Mn ²⁺ omitted	0.09
HCO ₃ ⁻ omitted	0.03
NADH omitted	0.03

K_m values for NAD⁺ and NADP⁺ were 0.50 and 0.56mM respectively.

Cation requirements. Like other 'malic' enzymes the cauliflower mitochondrial enzyme showed an absolute requirement for Mn²⁺, Co²⁺ or Mg²⁺. K_m and V_{max} values show that Mn²⁺ is the best cofactor, whereas Mg²⁺ is much less effective than either Co²⁺ or Mn²⁺ (Table 3). Slight activity was obtained with Ni²⁺ but other divalent metal ions were completely ineffective as cofactors for the reaction.

Some 'malic' enzymes require addition of K⁺ or NH₄⁺ for maximal activity (Ochoa *et al.* 1950; Sanwal & Smando, 1969). However, with the cauliflower mitochondrial enzyme, addition of K⁺ or NH₄⁺ to incubations devoid of these anions caused no increase in activity. At high concentrations K⁺ was slightly inhibitory.

Oxaloacetate decarboxylase activity. Incubations containing 2mM-Mn²⁺ and oxaloacetate at several concentrations were performed at various pH values between 4.0 and 7.0. For each set of conditions the rate of oxaloacetate decarboxylation, assayed by measurement of pyruvate formation, was determined with and without added cauliflower mitochondrial 'malic' enzyme. The rate of oxaloacetate

decarboxylation in the incubation mixtures containing enzyme was no greater than in those without enzyme. Addition of 1 mM-NAD⁺ to the incubation mixtures had no effect on the rate of oxaloacetate decarboxylation. These results show that, by contrast with most other 'malic' enzymes (Ochoa *et al.* 1950; Temperli *et al.* 1965; Takeo, 1969; Walker, 1962; Kun, 1963), the cauliflower mitochondrial enzyme has no appreciable oxaloacetate decarboxylase activity.

Reductive carboxylation of pyruvate. The 'malic' enzyme reaction is reversible and at high concentrations of CO₂ reductive carboxylation of pyruvate to malate can normally be observed (Walker, 1962; Kun, 1963). When the cauliflower mitochondrial

enzyme was incubated with pyruvate, HCO₃⁻, NADH and Mn²⁺ formation of malate occurred as expected (Table 4). Under optimum conditions (20 mM-pyruvate, 20 mM-HCO₃⁻, 0.5 mM-NADH and 2 mM-Mn²⁺) the rate of malate formation from pyruvate was one-twentyfifth of the rate of the reverse reaction assayed with 5 mM-malate, 1 mM-NAD⁺ and 2 mM-Mn²⁺.

Enzyme effectors. NADH was a powerful inhibitor of the oxidative decarboxylation reaction. The other nucleotides, NADPH, NADP⁺, ADP and ATP were less effective than NADH, and AMP and P_i gave no inhibition (Table 5). The inhibition by NADP⁺, ADP and ATP was partially reversed by raising the Mn²⁺ concentration from 2 to 5 mM, suggesting that these substances inhibit mainly by competing with the substrates for metal ion. The inhibition by NADH and NADPH was not reversed by raising the Mn²⁺ concentration. Further investigation of the inhibition by NADH showed it to be competitive with NAD⁺ at 5 mM-malate and non-competitive with malate at 1 mM-NAD⁺ (Fig. 2). It is probable that NADH competes with NAD⁺ at the active site of the enzyme.

Addition of low concentrations of CoA to incubation mixtures containing the crude 'malic' enzyme resulted in an increased rate of NAD⁺ reduction, which can be attributed to an activation of the 'malic' enzyme reaction. An alternative explanation for the CoA effect, involving oxidation of pyruvate in the presence of CoA, can be ruled out because no pyruvate dehydrogenase was detected in my preparations. In contrast with the crude enzyme the partially purified 'malic' enzyme showed no response to CoA. The absence of activation by CoA with the partially purified enzyme was due

Table 5. *Effect of nucleotides and P_i on 'malic' enzyme reaction*

The incubation mixtures contained 3 μmol of L-malate, 3 μmol of NAD⁺, 6 or 15 μmol of Mn²⁺, 60 μmol of TES, 54 μg of partially purified enzyme and inhibitor in a final volume of 3.0 ml. Final pH was 6.8, and incubation temperature 25°C. Reaction rates are expressed as a percentage of a control containing no inhibitor.

Inhibitor	Reaction rate (% of control)	
	2 mM-Mn ²⁺	5 mM-Mn ²⁺
0.5 mM-NADH	32	32
0.5 mM-NADPH	60	54
1 mM-AMP	97	98
1 mM-ADP	68	83
1 mM-ATP	76	88
1 mM-P _i	100	100
1 mM-NADP ⁺	88	94

Fig. 2. Inhibition of 'malic' enzyme by NADH. The incubation mixtures contained; in (a) 3 μmol of NAD⁺, various concentrations of L-malate, 6 μmol of Mn²⁺, 60 μmol of TES and 40 μg of partially purified enzyme; in (b) various concentrations of NAD⁺, 15 μmol of L-malate, 6 μmol of Mn²⁺, 60 μmol of TES and 36 μg of partially purified enzyme. Final volumes were 3.0 ml, final pH was 6.8, and incubation temp. 25°C. ○, No NADH; ●, 0.2 mM-NADH; ■, 0.4 mM-NADH. *v* is expressed in μmol of NADH/min.

Table 6. *Effect of CoA and salts on the 'malic' enzyme reaction*

The incubation mixtures contained 3 μ mol of L-malate, 3 μ mol of NAD⁺, 6 μ mol of Mn²⁺, 60 μ mol of TES, and 37 μ g of crude enzyme. Other additions are indicated below. Final volume was 3.0 ml, final pH 6.8, and incubation temperature 25°C.

Salt added	Reaction rate (μ mol of NADH/min)	
	No CoA	+10 μ M-CoA
None	0.011	0.026
20 mM-(NH ₄) ₂ SO ₄	0.020	0.020
20 mM-K ₂ SO ₄	0.019	0.018
20 mM-NH ₄ Cl	0.012	0.021
20 mM-KCl	0.011	0.024

Fig. 3. Effect of CoA on the 'malic' enzyme reaction at various concentrations of malate. The incubation mixtures contained various concentrations of L-malate, 3 μ mol of NAD⁺, 6 μ mol of Mn²⁺, 60 μ mol of TES and 60 μ g of crude enzyme in a total volume of 3.0 ml. Final pH was 6.8, and incubation temp. 25°C. ●, No CoA; ○, 1 μ M-CoA; □, 3.3 μ M-CoA; △, 10 μ M-CoA; ▲, 30 μ M-CoA.

to the presence of SO₄²⁻ ions in the preparations, since addition of 20 mM-ammonium sulphate or 20 mM-potassium sulphate to incubation mixtures containing the crude enzyme also resulted in a loss of activation by CoA (Table 6). This loss was accompanied by an increase in the activity of the enzyme in the absence of CoA. Potassium chloride and ammonium chloride had little effect on the 'malic' enzyme reaction rate. As expected removal of ammonium sulphate from the partially purified enzyme with Sephadex G-25 resulted in a decrease in enzyme activity and restoration of activation by CoA.

Fig. 4. Results in Fig. 3 replotted according to the Hill equation. Each curve was displaced vertically by a value, c , for the sake of clarity. ●, $c=0$; ○, $c=0.3$; □, $c=0.5$; △, $c=0.5$; ▲, $c=0.7$.

With the crude enzyme, at 1 mM-NAD⁺ and 2 mM-Mn²⁺, plots of reaction rate against malate concentration at various CoA concentrations showed that addition of CoA to the incubations causes a divergence from Michaelis-Menten kinetics. In the presence of CoA sigmoid curves were obtained (Fig. 3) and double-reciprocal plots of the results were non-linear. Hill plots (Atkinson, Hathaway & Smith, 1965) of the results showed that addition of CoA raises the interaction coefficient from 1.1 to 1.9 (Fig. 4). These results indicate that enzyme activation by CoA is accompanied by the appearance of co-operative binding of malate. At 5 mM-malate with NAD⁺ as the variable substrate, linear double-reciprocal plots of reaction rate against substrate concentration were obtained on addition of CoA to the incubation mixtures (Fig. 5). CoA had little effect on the K_m for NAD⁺ and no evidence of co-operative binding was obtained.

Other mitochondrial metabolites were also tested as possible effectors of the 'malic' enzyme reaction. Acetyl-CoA, aspartate, glutamate, alanine, pyruvate and the tricarboxylic acid-cycle acids, except for oxaloacetate, had no effect on the reaction. Oxaloacetate could not be tested on the partially purified enzyme because of the presence of malate dehydrogenase in the preparations. However, experiments with the more highly purified enzyme obtained from DEAE-Sephadex

Fig. 5. Effect of CoA on the 'malic' enzyme reaction at various concentrations of NAD⁺. The incubation mixtures contained 15 μ mol of L-malate, various concentrations of NAD⁺, 6 μ mol of Mn²⁺, 60 μ mol of TES and 60 μ g of crude enzyme in a total volume of 3.0 ml. Final pH was 6.8, and incubation temp. 25°C. ●, No CoA; ○, 1 μ M-CoA; Δ, 10 μ M-CoA. v is expressed as μ mol of NADH/min.

chromatography showed that oxaloacetate is an inhibitor of the 'malic' enzyme reaction. With 1 mM-malate, 1 mM-NAD⁺ and 2 mM-Mn²⁺ a 40% inhibition was given by 1 mM-oxaloacetate.

DISCUSSION

The results presented above suggest that the cauliflower mitochondrial 'malic' enzyme may be placed in the same category as the 'malic' enzymes from *Ascaris lumbricoides* and *Fasciola hepatica*. These enzymes have NAD⁺ as the principal cofactor but, in contrast with the NAD⁺-requiring malic enzymes from micro-organisms, show some activity with NADP⁺ and do not catalyse the decarboxylation of oxaloacetate (Saz & Hubbard, 1957; Pritchard & Schofield, 1968). No information is available about the cellular localization of the 'malic' enzyme in *Fasciola*, but like the cauliflower enzyme the *Ascaris* muscle enzyme occurs in the mitochondria and appears to be involved in the oxidation of malate (Papa *et al.* 1970). It seems possible that NAD⁺-requiring 'malic' enzymes will be found in the mitochondria of many eukaryotes that can utilize malate or other tricarboxylic acid-cycle acids for energy production.

The 'malic' enzymes from micro-organisms are subject to complex control mechanisms. In *Escherichia coli* the NADP⁺-requiring enzyme is inhibited allosterically by acetyl-CoA, oxaloacetate, NADH and NADPH (Sanwal & Smando, 1969), whereas the NAD⁺-requiring enzyme is inhibited by CoA and ATP and stimulated by aspartate

(Sanwal, 1970). The NAD⁺-requiring enzyme from *Streptococcus faecalis* is inhibited allosterically by nucleoside triphosphates and several glycolytic intermediates (London & Meyer, 1969b), and the *Neurospora* enzyme is inhibited by aspartate and oxaloacetate (Zink, 1967).

The inhibition by NADH and oxaloacetate and the activation by sulphate and CoA show that the cauliflower mitochondrial 'malic' enzyme also possesses complex regulatory properties. Detailed kinetic analysis of the influence of the effectors on the enzyme reaction is required before their mechanism of action can be established. However, the preliminary results reported here suggest that NADH may act as a straightforward competitive inhibitor, whereas stimulation by CoA involves complex subunit interactions.

The activation by CoA, especially at high malate concentrations, and the inhibition by NADH support the proposed role of the mitochondrial 'malic' enzyme in the utilization of malate or other tricarboxylic acid-cycle acids for energy production when glycolysis is suppressed (Macrae & Moorhouse, 1970). Thus the supply of pyruvate to the mitochondria from glycolysis will be reflected in the mitochondrial CoA concentrations, so that a cessation of glycolysis will lead to a build-up of free CoA in the mitochondria. Such a situation would cause a stimulation of the mitochondrial 'malic' enzyme leading to accelerated pyruvate formation from malate and a continuation of respiration by total oxidation of malate or one of the other tricarboxylic acid-cycle acids. In addition it has been suggested that the NAD⁺/NADH ratio, which reflects the ATP/ADP ratio by operation of the electron-transport chain, is a major regulatory factor in respiration by both animal (La Noue, Nicklas & Williamson, 1970) and plant (Coultate & Dennis, 1969) mitochondria. The comparatively small inhibition of the 'malic' enzyme by oxaloacetate is probably of no physiological significance, since mitochondrial concentrations of oxaloacetate are very low because of the equilibrium of the malate dehydrogenase reaction and the presence of transaminases.

REFERENCES

- Atkinson, D. E., Hathaway, J. A. & Smith, E. C. (1965). *J. biol. Chem.* **240**, 2682.
- Coultate, T. P. & Dennis, D. T. (1969). *Eur. J. Biochem.* **7**, 153.
- Kun, R. (1963). In *The Enzymes*, vol. 7, p. 149. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
- La Noue, K., Nicklas, W. J. & Williamson, J. R. (1970). *J. biol. Chem.* **245**, 102.
- London, J. & Meyer, E. Y. (1969a). *J. Bact.* **98**, 705.

- London, J. & Meyer, E. Y. (1969*b*). *Biochim. biophys. Acta*, **178**, 205.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Macrae, A. R. (1971). *Phytochemistry* (in the Press).
- Macrae, A. R. & Moorhouse, R. (1970). *Eur. J. Biochem.* **16**, 96.
- Nathan, H. A. (1961). *J. gen. Microbiol.* **25**, 415.
- Ochoa, S., Verga Salles, J. B. & Ortiz, P. J. (1950). *J. biol. Chem.* **187**, 863.
- Papa, S., Cheah, K. S., Rasmussen, H. N., Lee, I. Y. & Chance, B. (1970). *Eur. J. Biochem.* **12**, 540.
- Pritchard, R. & Schofield, P. (1968). *Biochim. biophys. Acta*, **170**, 63.
- Sanwal, B. D. (1970). *J. biol. Chem.* **245**, 1212.
- Sanwal, B. D. & Smando, R. (1969). *J. biol. Chem.* **244**, 1659.
- Saz, H. J. & Hubbard, J. A. (1957). *J. biol. Chem.* **225**, 921.
- Takeo, K. (1969). *J. Biochem., Tokyo*, **66**, 379.
- Temperli, A., Kunsch, U., Meyer, K. & Busch, I. (1965). *Biochim. biophys. Acta*, **110**, 630.
- Walker, D. A. (1962). *Biol. Rev.* **37**, 215.
- Zink, M. W. (1967). *Can. J. Microbiol.* **13**, 1217.