Control of the Citric Acid Cycle by Glyoxylate

MECHANISM OF THE INHIBITION BY OXALOMALATE AND γ-HYDROXY-α-OXOGLUTARATE

By A. RUFFO, E. TESTA*, A. ADINOLFI, G. PELIZZA* AND R. MORATTI

*Impresa Enzimologia C.N.R., c/o Institute of Biological Chemistry,
University of Pavia, Italy*

1. Hydroxyoxoglutarate was obtained by three methods: decarboxylation of oxalomalic acid, and synthesis from glyoxylate and pyruvate by using either Mg^{2+} or an enzyme from rat liver as catalysts. 2. The inhibitory effects of oxalomalate and hydroxyoxoglutarate upon aconitate hydratase, isocitrate dehydrogenase (NADP) and oxoglutarate dehydrogenase were investigated. 3. Oxalomalate at low concentrations (1 mM) inhibited almost completely both aconitate hydratase and isocitrate dehydrogenase. Hydroxyoxoglutarate also inhibited these enzymes, but at concentrations approximately tenfold that of oxalomalate. 4. Oxalomalate and hydroxyoxoglutarate, at the higher concentrations, inhibited oxoglutarate dehydrogenase to approximately the same extent. 5. It is suggested that the ability of glyoxylate to control reaction rates in the tricarboxylic acid cycle must in some degree be due to its condensation with oxaloacetate and pyruvate to form enzyme inhibitors.
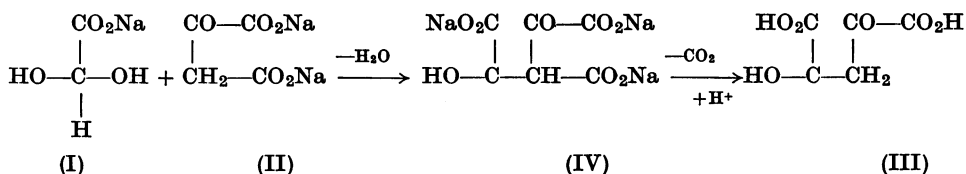
We previously showed that α-hydroxy-β-oxalosuccinate (oxalomalate) inhibits both aconitate hydratase [citrate (isocitrate) hydrolyase, EC 4.2.1.3] (Ruffo, Testa, Adinolfi & Pelizza, 1962) and isocitrate dehydrogenase (*threo*-D$_s$-isocitrate: NADP oxidoreductase, EC 1.1.1.42) (Ruffo & Adinolfi, 1963). Payes & Laties (1963) found that hydroxyoxoglutarate inhibits aconitate hydratase and isocitrate dehydrogenase of plant origin, as well as the oxidation of α-oxoglutarate by potato mitochondria. They emphasized that the inhibitor, which was obtained by incubation of glyoxylate (I) with oxaloacetate (II), was γ-hydroxy-α-oxoglutarate (III), and not oxalomalate (IV) (Scheme 1). They employed acidic solvents during the chromatographic isolation of the reaction product and

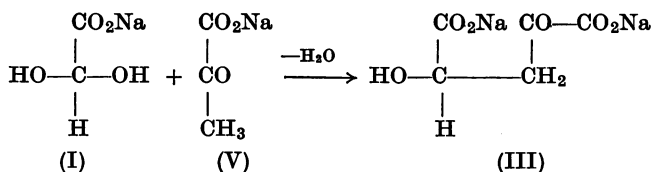* Present address: Dept. of Organic Synthesis, Lepetit S.p.A., Milano, Italy.

obtained hydroxyoxoglutarate. By avoiding acid conditions Ruffo *et al.* (1962) excluded the decarboxylation step and obtained oxalomalate. The question now arose whether the observed inhibitions were produced by oxalomalate or by its decarboxylation product hydroxyoxoglutarate.

We have prepared compound (III) by three methods: (1) decarboxylation of oxalomalate at pH 1, (2) condensation of glyoxylate (I) and pyruvate (V) in the presence of Mg^{2+} ions, and (3) condensation of (I) and (V) catalysed by an enzyme from rat liver (Scheme 2).

It is also possible to prepare (III) by a transamination reaction catalysed by an aminotransferase (Goldstone & Adams, 1962; Maitra & Dekker, 1963). We have compared the effect of γ-hydroxyoxoglutarate with that of oxalomalate on partially purified preparations of aconitate hydratase,

$$\underset{\text{(I)}}{\overset{\displaystyle \underset{|}{\overset{\text{CO}_2\text{Na}}{|}} }{\text{HO}-\overset{|}{\underset{\overset{|}{\text{H}}}{\text{C}}}-\text{OH}}} + \underset{\text{(II)}}{\overset{\displaystyle \overset{\text{CO}-\text{CO}_2\text{Na}}{\underset{|}{}}}{\text{CH}_2-\text{CO}_2\text{Na}}} \xrightarrow{-\text{H}_2\text{O}} \underset{\text{(IV)}}{\overset{\displaystyle \overset{\text{NaO}_2\text{C}\ \ \text{CO}-\text{CO}_2\text{Na}}{}}{\text{HO}-\text{C}-\text{CH}-\text{CO}_2\text{Na}}} \xrightarrow[+\text{H}^+]{-\text{CO}_2} \underset{\text{(III)}}{\overset{\displaystyle \overset{\text{HO}_2\text{C}\ \ \text{CO}-\text{CO}_2\text{H}}{}}{\text{HO}-\text{C}-\text{CH}_2}}$$

Scheme 1.

$$\underset{\text{(I)}}{\overset{\displaystyle \text{CO}_2\text{Na}}{\underset{\displaystyle \text{H}}{\text{HO—C—OH}}}} \ + \ \underset{\text{(V)}}{\overset{\displaystyle \text{CO}_2\text{Na}}{\underset{\displaystyle \text{CH}_3}{\text{CO}}}} \ \xrightarrow{\ -\text{H}_2\text{O}\ } \ \underset{\text{(III)}}{\overset{\displaystyle \text{CO}_2\text{Na} \quad \text{CO—CO}_2\text{Na}}{\underset{\displaystyle \text{H}}{\text{HO—C————CH}_2}}}$$

Scheme 2.

isocitrate dehydrogenase and oxoglutarate dehydrogenase (2-oxoglutarate: lipoate oxidoreductase, EC 1.2.4.2).

EXPERIMENTAL

Materials. NAD+ grade III, and NADP+ (sodium salt), were 98% pure preparations from Sigma Chemical Co. (St Louis, Mo., U.S.A.); coenzyme A was a 75% pure preparation from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), or an 80% pure preparation from Sigma Chemical Co.; Amberlite IR-120 was obtained from British Drug Houses Ltd. (Poole, Dorset). Isocitrate dehydrogenase (NADP) was an analytical grade preparation from Boehringer containing 1·8 units/mg. (1 unit corresponds to 1 μmole of substrate transformed in 1 min.). Trisodium DL-isocitrate was a Sigma preparation (93–98% pure); *cis*-aconitic acid was obtained from Fluka A.G. (Buchs SG, Switzerland); trisodium citrate dihydrate, 99% pure, was from E. Merck A.G. (Darmstadt, Germany); monopotassium oxoglutarate, sodium pyruvate and sodium glyoxylate monohydrate were from Sigma or from Fluka. Tris was a Sigma product. All the other reagents were from C. Erba (Milan, Italy).

Analytical methods. Ketonic functions were determined as 2,4-dinitrophenylhydrazones either by the colorimetric method of Elgart & Nelson (1941) or with the chromatographic method previously described (Ruffo *et al.* 1962). Glyoxylate (as aldehyde) was determined by the procedure of Kramer, Klein & Baselice (1959). Citrate was assayed by the colorimetric method of Ettinger, Goldbaum & Smith (1952) and *cis*-aconitate by its extinction at 240mμ (Ruffo *et al.* 1962). Infrared spectra were determined in a Nujol mull with a Perkin–Elmer model 12 C spectrophotometer fitted with a NaCl prism. Melting points (uncorrected) were determined on a Kofler apparatus.

Preparation of enzymes: assay methods. Aconitate hydratase was prepared from pig heart according to Anfinsen (1955) and the activity was determined with either *cis*-aconitate or citrate as substrate (Ruffo *et al.* 1962).

Isocitrate dehydrogenase was prepared from pig heart according to Ochoa (1955), the procedure being interrupted at the first (NH₄)₂SO₄ fractionation step (50–60% saturation); in some experiments the Boehringer preparation was used. The activity was determined as described by Ruffo & Adinolfi (1963).

Oxoglutarate dehydrogenase was purified according to Sanadi, Littlefield & Bock (1952) and the activity was determined as reported by Hift, Ouellet, Littlefield & Sanadi (1953), except that phosphorylating enzyme and GDP were omitted and the concentration of CoA was increased to 0·5 μmole.

Preparation of inhibitors. Sodium oxalomalate trihydrate was prepared as described by Ruffo *et al.* (1962). γ-Hydroxy-α-oxoglutarate was prepared in three ways.

Method 1. Sodium oxalomalate (1 g.) was dissolved in 25 ml. of water, saturated with N₂, and acidified with 10% (w/v) HCl to pH 1. After 5 min. at 0° 2 N-NaOH was added to bring the pH to about 6. Then 40 ml. of 10% (w/v) BaCl₂ was added, and the precipitate was filtered off. The filtrate was diluted with 100 ml. of 95% (v/v) ethanol and left for 2 hr. at 2°. The precipitate was collected by filtration, washed with absolute ethanol and dried *in vacuo*. The yield was approx. 95%, as determined by the transformation of the acid into its 2,4-dinitrophenylhydrazone and by microanalysis as described by Ruffo *et al.* (1962).

Method 2. Freshly distilled pyruvic acid (2 g.) and 2·6 g. of sodium glyoxylate monohydrate were dissolved in 60 ml. of water. The pH was adjusted to 6 with N-NaOH and 6 g. of MgCl₂,6H₂O was added. The pH was again adjusted to 6 and the solution was held at 40° for 4 hr. The rate of condensation was followed by determining the rate of the disappearance of the aldehyde group of glyoxylate. The solution was treated, at room temperature, with an equal volume of 10% (w/v) BaCl₂; 0·2 vol. of 95% (v/v) ethanol was added, and the solution was left for 2 hr. at 2°. The precipitate was collected on a Buchner filter and dried. The yield of product (*a*) was 4·2 g. The filtrate was again treated with an equal volume of 95% ethanol to yield 1·4 g. of product (*b*). Further dilution of the filtrate with an equal volume of ethanol gave 0·8 g. of product (*c*). This last fraction, which had the highest content of γ-hydroxyoxoglutarate, was suspended in 16 ml. of water, dissolved by adding 1·2 ml. of 10% (w/v) HCl, and neutralized to pH 6 with N-NaOH. To this solution 4 ml. of 95% ethanol was added and, after 2 hr. at 2°, 0·36 g. of fraction (*d*) was collected. The filtrate was diluted with 80 ml. of 95% ethanol and kept for 2 hr. at 2°, when 360 mg. of compound (*e*) was obtained. This last was used for investigating the effect of (III) on enzymic activities. On the basis of colorimetric analysis of the ketonic function, the purities of the different fractions were: (*a*) 10%; (*b*) 25%; (*c*) 60%; (*d*) 30%; (*e*) 89%. Chromatographic analysis of the dinitrophenylhydrazone of (*e*) showed only one spot with the same R_F as that of the product of decarboxylation of oxalomalate. The melting point of the dinitrophenylhydrazone (204–205°), as well as the microanalytical data and the i.r. spectrum, were in agreement with the results obtained by Ruffo *et al.* (1962). Analysis of fraction (*e*) gave the following results. Found: C, 19·9; H, 1·5; Ba, 46·1; Calc. for $C_5H_4O_6Ba$: C, 20·1; H, 1·4; Ba, 46·1%. The infrared spectrum of this compound showed typical bands at 3300 cm.⁻¹ (OH stretching), 1700 cm.⁻¹ (C=O stretching), 1600 cm.⁻¹ (CO₂⁻ stretching) and 1080 cm.⁻¹ (C–O stretching).

Method 3. Pyruvate and glyoxylate were condensed

enzymically by the method of Kuratomi & Fukunaga (1963). The product was transformed into the phenylhydrazone and chromatographed on paper. Only one spot was observed and its R_F was equal to that of the phenylhydrazone of the compound obtained from decarboxylation of oxalomalate.

Preparation of inhibitor solutions. Sodium oxalomalate was dissolved in cold water immediately before use. Hydroxyoxoglutarate (III) was obtained either as the barium salt or the calcium salt. The barium salt was transformed into the sodium salt by dissolving it in 0·2 N-HCl and adding the stoicheiometric amounts of Na_2SO_4. The $BaSO_4$ was centrifuged off at low temperature, and the supernatant was neutralized to pH 7·4. The calcium salt was transformed into the sodium salt by stirring its aqueous solution with washed Amberlite IR-120 (H⁺ form) for 10 min., filtering off the resin and neutralizing the filtrate with NaOH.

RESULTS

Chemical condensation of glyoxylate with pyruvate. The effect of pH on the reaction rate, in the presence and in the absence of magnesium chloride, is shown in Fig. 1. Preliminary results showed that the maximum rate was obtained at 40°. Though Mg^{2+} did not influence the rate at pH 3, it produced marked increments at pH 6 and 7·4.

Inhibitory effects of oxalomalate and γ-hydroxy-α-oxoglutarate. (a) Inhibition of aconitate hydratase. In these experiments the reaction was started by addition of substrate. The effects of the inhibitors on the enzyme, with 10 μmoles of *cis*-aconitate as
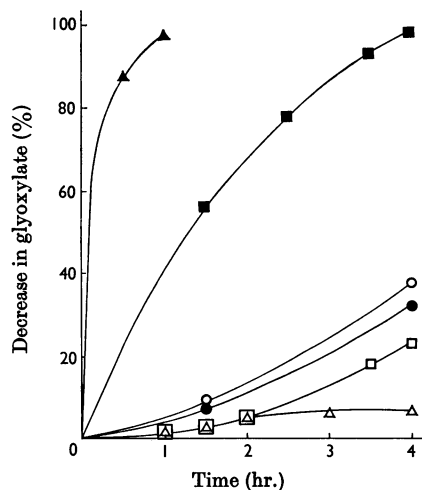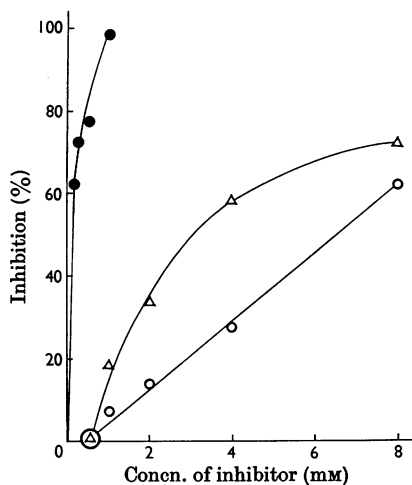

Fig. 2. Effect of oxalomalate and hydroxyoxoglutarate on aconitate hydratase. The incubation mixtures contained, in a final volume 4 ml.: 10 μmoles of *cis*-aconitate; 0·2 ml. of enzyme solution (1–2 mg. of protein/ml.); 2·5 ml. of 0·1 M-phosphate–KCl buffer, pH 7·4 (0·9% with respect to KCl), together with inhibitors. ●, Effect of oxalomalate; ○ and △, effect of hydroxyoxoglutarate prepared by methods 1 and 2 respectively. Mixtures were incubated for 30 min. at 37° and deproteinized with 3 ml. of freshly prepared 5% (w/v) tungstic acid.
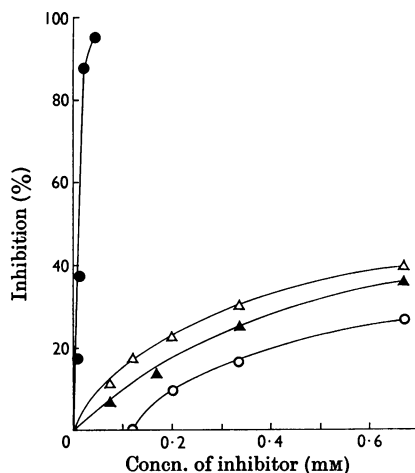

Fig. 1. Disappearance of glyoxylate during its condensation with pyruvate at 40°. The incubation mixture contained, in 10 ml., 314 mg. of sodium glyoxylate and 241 mg. of pyruvic acid. ○, pH 3; ●, pH 3, in the presence of 0·35 M-MgCl₂; □ pH adjusted to 6 with N-NaOH; ■, pH 6, in the presence of 0·35 M-MgCl₂; △, pH adjusted to 7·4 with N-NaOH; ▲, pH 7·4, in the presence of 0·35 M-MgCl₂.

Fig. 3. Effect of oxalomalate and hydroxyoxoglutarate on aconitate hydratase. Incubation mixtures were prepared in 10 mm. silica cuvettes by mixing 0·2 ml. of enzyme solutions (1–2 mg. of protein/ml.), 2 ml. of 0·05 M-phosphate buffer, pH 7·4, together with inhibitors. ●, Effect of oxalomalate. ○, △, ▲, Effect of three samples of hydroxyoxoglutarate prepared by methods 1, 2 and 3 respectively. The reaction was started by the addition of 15 μmoles of trisodium citrate, and the volume was adjusted to 3 ml. with water. Readings at 240 mμ were taken for 3 min. and the change in extinction (ΔE) between 30 and 60 sec. was taken as a measure of enzyme activity.

substrate, are shown in Fig. 2. At a concentration of 1 mM, hydroxyoxoglutarate exerted a negligible inhibition whereas oxalomalate decreased the activity by more than 90%. At concentrations lower than 1 mM, hydroxyoxoglutarate had no effect. With citrate as substrate, the rate of *cis*-aconitate formation was followed directly in a spectrophotometer cell and it was thus possible to investigate the effect of lower concentrations of inhibitors during the first few minutes of the reaction (Fig. 3). Again there was a very marked difference between the inhibitory activity of oxalomalate and that of the three samples of hydroxyoxoglutarate. Other experiments showed that the inhibition produced by both inhibitors was competitive.

(*b*) Inhibition of isocitrate dehydrogenase. Previous experiments (Ruffo & Adinolfi, 1963) showed that preincubation of oxalomalate with this enzyme increased the inhibition. Therefore we mixed in the spectrophotometer cell all the reactants within 3 min. and then we started the reaction with the substrate (Fig. 4). Again, oxalomalate produced a much higher inhibition than hydroxyoxoglutarate. It was not possible, under our experimental conditions, to establish whether the inhibitions were of the competitive type.

(*c*) Inhibition of oxoglutarate dehydrogenase. Preliminary experiments showed that this enzyme was less sensitive to the inhibitors and, in some experiments, hydroxyoxoglutarate underwent oxidation. Therefore in the following experiments the inhibitors were preincubated with the enzyme for 15 min. at room temperature and a blank sample containing all reactants except substrate was taken as control. The reaction was started by the addition of CoA. Fig. 5 shows that there was no difference between the inhibitory effects of hydroxyoxoglutarate and oxalomalate. With both inhibitors the inhibition was competitive.

DISCUSSION

As an inhibitor of aconitate hydratase and isocitrate dehydrogenase, oxalomalate is about ten times as effective as hydroxyoxoglutarate. This difference was particularly evident with aconitate hydratase. With oxoglutarate dehydrogenase there was no significant difference between the effects of the two inhibitors. Similarly, in the oxidation of
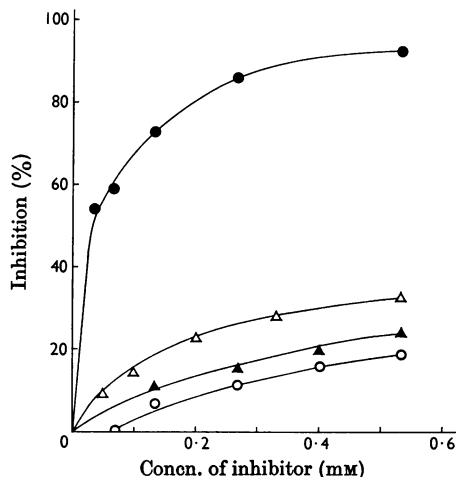

Fig. 4. Effect of oxalomalate and hydroxyoxoglutarate on isocitrate dehydrogenase. Incubation mixtures were prepared in 10 mm. silica cuvettes by mixing 0·3 ml. of 0·25 M-tris buffer, pH 7·4, 0·1 ml. of enzyme solution (0·5 mg. of protein/ml.), 0·1 ml. of 0·018 M-MnCl₂ and 0·4 ml. of 1 mM-NADP⁺. ●, Effect of oxalomalate; ○, △, ▲, effects of hydroxyoxoglutarate prepared by methods 1, 2 and 3 respectively. The mixture was adjusted to 2·9 ml. with water, and the reaction was started by addition of 0·1 ml. of 8 mM-DL-isocitrate. Readings were taken at 340 mμ and ΔE for the interval between 30 and 60 sec. was taken as a measure of enzyme activity.
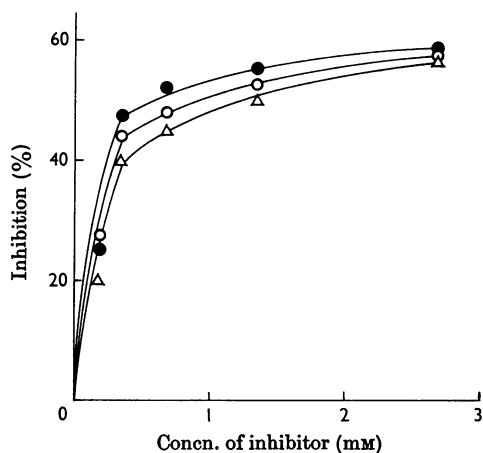

Fig. 5. Effect of oxalomalate and hydroxyoxoglutarate on oxoglutarate dehydrogenase. The enzyme was preincubated with inhibitors by mixing them in a 10 mm. silica cuvette with 0·02 ml. of enzyme solution (3–4 mg. of protein/ml.) and 1 ml. of 0·1 M-potassium phosphate buffer, pH 7·5. After 15 min., were added, in order: 1 μmole of NAD⁺; 10 μmoles of MgCl₂; 10 μmoles of cysteine; 5 μmoles of oxoglutarate; 0·5 μmole of CoA, to start the reaction. The final volume was adjusted to 3 ml. Readings were taken at 340 mμ for 3 min. and ΔE for the interval 0–20 sec. was measured. ●, Effect of oxalomalate; ○, △, effect of hydroxyoxoglutarate prepared by methods 1 and 2 respectively.

oxoglutarate by rat-liver mitochondria, Ruffo, Malcovati & Adinolfi (1965) found that the addition of either oxalomalate or hydroxyoxoglutarate produced comparable inhibitions of uptake of oxygen.

Contrary to Payes & Laties (1963), the initial product of the reaction between glyoxylate and oxaloacetate at pH 7·4 is oxalomalate, which we have isolated in the pure state as its trisodium salt (Ruffo et al. 1962). Because oxalomalate has a much higher inhibitory activity than hydroxyoxoglutarate, it is clear that oxalomalate, rather than its decomposition product, was the effective inhibitor when oxalomalate was added to our preparations of aconitate hydratase and isocitrate dehydrogenase.

It remains to consider the differences in inhibitory activity of the three preparations of hydroxyoxoglutarate used in this work. These variations might be due to variations in the ratio between the keto and enol forms or to partial transformation of hydroxyoxoglutarate into its lactone (Goldstone & Adams, 1962). In addition, the chemical method of preparation would yield an optically inactive product whereas the enzymic synthesis would presumably lead to one optical isomer.

Since both oxalomalate and hydroxyoxoglutarate may be formed, under physiological conditions of temperature and pH, by condensation of glyoxylate with either oxaloacetate or pyruvate, formation of these inhibitors probably plays an important role in the regulation of the tricarboxylic acid cycle.

REFERENCES

Anfinsen, C. B. (1955). In *Methods in Enzymology*, vol. 1, p. 695. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Elgart, S. & Nelson, N. (1941). *J. biol. Chem.* **138**, 443.

Ettinger, R. H., Goldbaum, L. R. & Smith, L. H. (1952). *J. biol. Chem.* **199**, 513.

Goldstone, A. & Adams, E. (1962). *J. biol. Chem.* **237**, 3476.

Hift, H., Ouellet, L., Littlefield, J. W. & Sanadi, D. R. (1953). *J. biol. Chem.* **204**, 565.

Kramer, D. N., Klein, N. & Baselice, R. A. (1959). *Analyt. Chem.* **31**, 250.

Kuratomi, K. & Fukunaga, K. (1963). *Biochim. biophys. Acta*, **78**, 617.

Maitra, U. & Dekker, E. E. (1963). *J. biol. Chem.* **238**, 3660.

Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 699. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Payes, B. & Laties, G. G. (1963). *Biochem. biophys. Res. Commun.* **10**, 460.

Ruffo, A., Testa, E., Adinolfi, A. & Pelizza, G. (1962). *Biochem. J.* **85**, 588.

Ruffo, A. & Adinolfi, A. (1963). *Biochem. J.* **89**, 50 P.

Ruffo, A., Malcovati, M. & Adinolfi, A. (1965). In *Regulation of Metabolic Processes in Mitochondria*, B.B.A. Library vol. 7, p. 518. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.

Sanadi, D. R., Littlefield, J. W. & Bock, R. M. (1952). *J. biol. Chem.* **197**, 851.