The mechanism of tyrosinase-catalysed oxidative decarboxylation of α -(3,4-dihydroxyphenyl)-lactic acid

Manickam SUGUMARAN,* Hemalata DALI† and Victor SEMENSI

Department of Biology, University of Massachusetts at Boston, Harbor Campus, Boston, MA 02125, U.S.A.

Mushroom tyrosinase, which is known to catalyse the conversion of *o*-diphenols into *o*-benzoquinones, has been shown to catalyse the oxidative decarboxylation of 3,4-dihydroxymandelic acid [Sugumaran (1986) *Biochemistry* **25**, 4489–4492]. To account for this unusual reaction, a quinone methide intermediate has been proposed. Since all attempts to trap this intermediate ended in vain, mechanistic studies were designed to support the formation of this transient product. Replacement of the α -proton in 3,4-dihydroxymandelic acid with a methyl group generates α -(3,4-dihydroxyphenyl)-lactic acid, the enzymic oxidation of which should produce 3,4-dihydroxyacetophenone as the end product if the oxidative decarboxylation proceeds through the quinone methide intermediate. Accordingly, chemically synthesized α -(3,4-dihydroxyphenyl)-lactic acid on enzymic oxidation produced 3,4-dihydroxyacetophenone as the major isolatable product. Non-steady-state kinetic analysis of the enzyme reaction attested to the transient formation of the conventional quinone product. Thus the enzymic oxidation of α -(3,4-dihydroxyphenyl)-lactic acid seems to generate the conventional quinone, which, owing to its instability, is rapidly decarboxylated to yield the transient quinone methide. The coupled dieneone-phenol re-arrangement and ketol-enol tautomerism transforms the quinone methide into 3,4-dihydroxyacetophenone.

INTRODUCTION

Tyrosinase (EC 1.14.18.1), a widely distributed mono-oxygenase that is responsible for the biosynthesis of melanin pigments in a vast array of organisms, usually catalyses the conversion of monophenols into *o*-diphenols and oxidation of diphenols to quinones (Lerch, 1983; Robb, 1984). A few years ago we reported an unusual oxidative decarboxylation of 3,4-dihydroxymandelic acid (DHMA) to dihydroxybenzaldehyde (DHBald) catalysed by mushroom tyrosinase (Sugumaran, 1986). We could not detect the formation of a stable conventional quinone product during the steady state of the reaction; therefore we proposed a direct enzyme-catalysed oxidative decarboxylation of DHMA to an unstable quinone methide. The quinone methide formed, owing to its instability, is rapidly aromatized to DHBald by a coupled dieneone-phenol re-arrangement and keto-enol tautomerism (Scheme 1). After our studies, two different groups of workers re-examined this reaction using different techniques and demonstrated the production of the conventional quinone product during both enzymic and chemical oxidation of DHMA (Ortiz *et al.*, 1988; Cabanes *et al.*, 1988; Bouheroum *et al.*, 1989). The mandeloquinone formed seems to be highly unstable and generates DHBald probably via the proposed quinone methide intermediate.

Although the formation of quinone intermediate is certain and is consistent with the mode of action of tyrosinase, evidence for quinone methide formation in this reaction has not been obtained so far. If quinone methide is indeed formed by the unimolecular decarboxylation of mandeloquinone, then substitution of the α -proton in DHMA with a methyl group would generate 3,4-dihydroxyacetophenone (DHAP) as the major product (Scheme 1; R = CH₃). To test this possibility, we chemically synthesized α -(3,4-dihydroxyphenyl)-lactic acid (DHPLA) and examined its oxidation by tyrosinase. The results of our studies support both the transient quinone formation and unstable quinone methide production during mushroom-tyrosinase-catalysed oxidation of DHPLA.

MATERIALS AND METHODS

Synthesis of DHPLA (Scheme 2)

A mixture of 3,4-dimethoxyacetophenone (5.4 g, 30 mmol), trimethylsilyl cyanide (4.2 ml, 33 mmol) and ZnI₂ (150 mg) in

Scheme 1. Proposed mechanism for oxidative decarboxylation of DHMA by tyrosinase

Tyrosinase-catalysed oxidative decarboxylation of DHMA (A, R = H) generates DHBald (D, R = H). Initially we proposed a direct oxidative decarboxylation of A to the quinone methide (C, R = H) and its transformation into D to account for this reaction, but other workers (see the text) have shown that mandeloquinone (B) is an intermediate in this reaction.

Abbreviations used: DHBald, 3,4-dihydroxybenzaldehyde; DHAP, 3,4-dihydroxyacetophenone; DHMA, 3,4-dihydroxymandelic acid; DHPLA, α -(3,4-dihydroxyphenyl)-lactic acid.

* To whom correspondence should be addressed.

† Deceased 6 May 1990.

Scheme 2. Scheme for the synthesis of DHPLA

1, 3,4-Dimethoxyacetophenone; 2, trimethylsilyl derivative; 3, cyanohydrin of 3,4-dimethoxyacetophenone; 4, α -(3,4-dimethoxyphenyl)-lactic acid; 5, *O*-acetyl- α -(3,4-dimethoxyphenyl)-lactic acid; 6, DHPLA.

40 ml of methylene chloride was stirred under N_2 at room temperature for 2 h. Removal of solvent on a rotary evaporator gave an oil (7.8 g) of 3,4-dimethoxyacetophenone trimethylsilyl cyanide. The crude silane derivative was stirred with 3 M-HCl (45 ml) at room temperature for 2 h. The cyanohydrin formed was extracted with diethyl ether (3×50 ml) and the extract was dried over anhydrous $MgSO_4$. Removal of solvent gave nearly quantitative yield of cyanohydrin (5.8 g), m.p. 64–65 °C. I.r. (Nujol mull): $\nu = 3380$ (OH), 1600, 1520, 1400, 1320, 1260, 1140, 1030, 950, 855, 815 and 770 cm^{-1} . 1H n.m.r. [2H]chloroform: δ (p.p.m.) 1.85 (3H, singlet, CH_3), 3.85 (6 H, singlet OCH_3), 4.30 (1H, broad, OH) and 6.60–7.30 (3H, multiplet, ArH).

A solution of cyanohydrin (0.95 g, 4.7 mmol) in 40 ml of methanol was saturated with HCl gas at 0 °C and then allowed to warm up to room temperature. After 2 h, the solvent was removed on a rotary evaporator. The product was taken up in water (15 ml) and stirred for an additional 2 h. NaOH pellets (0.5 g) were added to this mixture and the contents stirred for 1 h. After acidification, the α -(3,4-dimethoxyphenyl)-lactic acid formed was extracted with diethyl ether (3×50 ml) and dried over anhydrous $MgSO_4$. Removal of ether *in vacuo* and recrystallization in benzene gave a white solid of α -(3,4-dimethoxyphenyl)-lactic acid (0.65 g; 63% yield), m.p. 102–104 °C. (Found: C, 58.33; H, 6.26. Calc. for $C_{11}H_{14}O_5$: C, 58.41; H, 6.19%. M_r 226.23.) I.r. (Nujoll mull): $\nu = 3300$ (OH), 2600 (broad, CO_2H), 1700 ($C=O$), 1370 (CH_3), 1250 (OCH_3) and 1130 cm^{-1} (OH). 1H n.m.r. ([2H]chloroform): δ (p.p.m.) 1.80 (3H, singlet, CH_3), 3.80 (6H, singlet, OCH_3), 6.53–7.30 (3H, multiplet, ArH) and 8.00 (2H, broad, CO_2H+OH).

A mixture of α -(3,4-dimethoxyphenyl)-lactic acid (0.678 g, 3 mmol), acetic anhydride (6 ml) and $FeCl_3$ (60 mg) was stirred at 0 °C for 0.5 h and poured on ice (10 g). The acetyl derivative thus obtained was extracted with diethyl ether (3×50 ml). The ether extract was washed with water followed by saturated NaCl solution and dried over anhydrous $MgSO_4$. Removal of diethyl ether yielded a colourless oil (0.78 g; yield 95%), which gave a single spot on silica-gel t.l.c. (R_f 0.36 in ethyl acetate). I.r. (Nujoll mull): $\nu = 3400$ –2400 (broad, CO_2H), 1745, 1705 ($C=O$), 1610, 1600, 1520, 1410, 1260 (OCH_3), 1120 (OH), 1030, 830 and 770 cm^{-1} . 1H n.m.r. ([2H]chloroform): δ (p.p.m.) 2.00 (3H, singlet, CH_3), 2.10 (3H, singlet, CH_3), 3.80 (6H, doublet, OCH_3), 6.53–7.53 (3H, multiplet, ArH) and 10.63 (1H, singlet, CO_2H).

A solution of *O*-acetyl- α -(3,4-dimethoxyphenyl)-lactic acid (0.73 g, 2.7 mmol) in 50 ml of methylene chloride was slowly added to a stirred solution of 0.25 M-boron tribromide in

methylene chloride (30 ml) at -80 °C over a period of 0.5 h. The mixture was allowed to warm up to room temperature in 1 h and was treated with ice-cold water (20 ml). The pH of the solution was adjusted to 6 and the contents were freeze-dried. Bio-Gel P-2 column chromatography of the residue with 0.2 M-acetic acid gave a white solid of DHPLA (150 mg; yield 33%; overall yield approx. 20%), m.p. 58–60 °C. I.r. (neat): $\nu = 3500$ –2300 (broad, CO_2H+OH), 1720 ($C=O$), 1600, 1525, 1510, 1440, 1360, 1260, 1120, 1020, 950, 870, 815 and 785 cm^{-1} . 1H n.m.r. (2H_2O): δ (p.p.m.) 1.73 (3H, singlet, CH_3) and 6.30–7.15 (3H, multiplet, ArH).

Large-scale reaction

A reaction mixture containing DHPLA (260 mg) and mushroom tyrosinase (6 mg) in 400 ml of 50 mM-sodium phosphate buffer, pH 6.0, was incubated at room temperature for 4 h. At the end of this period, the contents were freeze-dried and the residue was chromatographed on a Bio-Gel P-2 column with 0.2 M-acetic acid as the eluent. The fractions containing the required product were pooled and concentrated by freeze-drying to obtain DHAP as a white solid (130 mg; isolated yield 65%), m.p. 114–115 °C (standard m.p. 114–115 °C)

Other procedures

H.p.l.c. analysis of the reaction mixtures was carried out as outlined by Sugumaran (1986). U.v. and visible spectra were recorded by using a Milton Roy spectronic 3000 array spectrophotometer. I.r. spectra were obtained from a Perkin-Elmer model 137 spectrophotometer and 1H -n.m.r. spectra were recorded from a 60 MHz Perkin-Elmer model R-24 spectrometer.

Chemicals

Mushroom tyrosinase (specific activity 4000 units/mg) was obtained from Sigma Chemical Co. 3,4-Dimethoxyacetophenone was procured from Chemical Dynamics Corp., South Plainfield, NJ, U.S.A. DHAP was purchased from Pfaltz and Bauer, Stanford, CT, U.S.A. Other chemicals were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

RESULTS

Mushroom tyrosinase is known to exhibit wide substrate specificity and to oxidize a number of *o*-diphenolic compounds (Robb, 1984; Sugumaran, 1986). Accordingly the fungal enzyme

Fig. 1. U.v.-absorbance-spectral changes associated with the enzymic oxidation of DHPLA

A reaction mixture containing 0.2 mM-DHPLA and 10 μ g of mushroom tyrosinase in 1 ml of (a) 100 mM-sodium phosphate buffer, pH 6.0 (1.5 min scans), or (b) 100 mM-Tris/HCl buffer, pH 8.0 (1 min scans), was incubated at room temperature and the change in absorbance was recorded at regular intervals.

Fig. 2. Time course of quinone formation from DHPLA during the initial phase of the reaction

A reaction mixture (500 μ l) containing 250 μ g of mushroom tyrosinase, 20 mM-DHPLA in 100 mM-sodium phosphate buffer, pH 6.0, was incubated at room temperature and the absorbance change at 400 nm due to quinone formation was continuously monitored. Note the rapid initial burst in quinone formation and the reaching of the steady-state level. Inset: visible spectrum of DHPLA quinone formed in the reaction mixture at about 40 s.

readily oxidized chemically synthesized DHPLA. Fig. 1 shows the u.v.- and visible-spectral changes accompanying the enzymic oxidation of DHPLA at two different pH values. As is evident, the spectral changes failed to provide evidence for the formation and accumulation of quinone (as judged by the absence of absorbance increase at about 400 nm) in the reaction mixture. However, rapid spectral changes occurring in the u.v. region indicate that DHPLA is being converted into a product that exhibits absorbance at both 280 nm and 320 nm (at pH 6.0).

Fig. 3. H.p.l.c. analysis of the tyrosinase/DHPLA reaction mixture

A reaction mixture (1 ml) containing 10 μ g of mushroom tyrosinase and 2 mM-DHPLA in 100 mM-sodium phosphate buffer, pH 6.0, was incubated at room temperature, and 20 μ l portions of the reaction mixture were subjected to h.p.l.c. analysis on a Nova Pak Phenyl cartridge column (4 μ m particle size; 8 mm $\times$ 100 mm) (Waters Associates, Milford, MA, U.S.A.) with isocratic elution with 50 mM-acetic acid containing 0.2 mM-sodium octanesulphonate in 30% (v/v) methanol at a flow rate of 1 ml/min. (a) Experimental, zero-time reaction; (b) experimental, 14 min reaction; (c) authentic DHAP.

These spectral changes were similar to those reported for the tyrosinase-catalysed oxidation of DHMA (Sugumaran, 1986), and hence the course of tyrosinase-catalysed oxidation of DHPLA is apparently the same as that of DHMA oxidation.

In the case of DHMA, the quinone formed was found to be very unstable (Ortiz *et al.*, 1988; Cabanes *et al.*, 1988; Bouheroum *et al.*, 1989). A similar situation might exist even in the case of DHPLA. Therefore attempts were made to monitor the quinone formation specifically. Under the standard assay conditions used for Fig. 1, quinone formation in the reaction mixture could not be readily seen even during the initial phase of the reaction. However, use of large amounts of reactants and monitoring the initial phase of enzymic oxidation provided evidence for the transient generation of quinone.

Fig. 2 shows the time course of absorbance changes occurring at 400 nm during the initial phase of tyrosinase-catalysed oxidation of DHPLA. It is evident that a species absorbing at 400 nm is rapidly formed and reaches a steady-state concentration in the reaction mixture. The visible spectrum of this intermediate shown in the inset to Fig. 2 corresponded closely to the spectra of related quinones (Sugumaran, 1986). Thus tyrosine-catalysed oxidation of DHPLA seems to produce the conventional quinone that rapidly decomposes to the product exhibiting absorbance at 280 and 320 nm (Fig. 1). In order to characterize the product, h.p.l.c. studies were carried out.

H.p.l.c. analysis (Fig. 3) of the DHPLA/tyrosinase reaction mixture indicated the formation of a single product in near-quantitative yield, whose retention time and elution profile

Fig. 4. U.v. spectrum of (A) enzymic product and (B) authentic DHAP in water

Fig. 5. I.r. spectrum of (a) enzymic product and (b) authentic DHAP

matched that of an authentic sample of DHAP. On co-chromatography in different solvent systems, both the enzymic product and authentic DHAP were eluted as a single symmetrical peak (results not shown). Moreover, these two compounds showed the same absorbance spectrum and exhibited absorbance at 290 and 311 nm (Fig. 4), indicating that tyrosine-catalysed oxidation of DHPLA results in the production of DHAP. Accordingly, from a large-scale reaction mixture a product was isolated whose spectral properties matched that of an authentic sample of DHAP in every respect. The i.r. spectrum of the isolated product matched peak to peak with that of the authentic DHAP (Fig. 5), and the ^1H -n.m.r. spectrum (Fig. 6) attested that these two compounds are one and the same. Thus the tyrosine-catalysed oxidation of DHPLA indeed produces DHAP as the stable product, analogous to the conversion of DHMA into DHBald (Sugumaran, 1986).

The DHPLA quinone transiently formed in the reaction mixture can either be directly decarboxylated to the quinone methide tautomer of DHAP or be indirectly converted into DHAP by the quinone-mediated side chain oxidation of DHPLA. Since quinones are good oxidizing agents, the possibility of non-enzymic oxidation of the DHPLA side chain by the enzymically generated mandeloquinone seemed to be a likely reaction. However, DHPLA, being a tertiary alcohol, is not directly

Fig. 6. N.m.r. spectrum of (a) enzymic product and (b) authentic DHAP in DMSO-d_6 after $^2\text{H}_2\text{O}$ exchange

susceptible to oxidation at the side chain. Moreover, exogenously supplied 1-(3,4-dihydroxyphenyl)ethanol was oxidized by tyrosinase to the conventional quinone product instead, ruling out the possibility that DHPLA quinone is oxidizing the side chain of DHPLA. Hence, it is likely that DHPLA quinone is directly decarboxylated to generate the quinone methide by a unimolecular reaction (Scheme 1, $\text{R} = \text{CH}_3$).

DISCUSSION

Novel oxidative decarboxylation of DHMA to DHBald was first reported from this laboratory (Sugumaran, 1986). On the basis of the available evidence, we proposed a direct tyrosinase-catalysed oxidative decarboxylation of DHMA to a quinone methide and subsequent transformation of the quinone methide to account for the observed reaction. However, in subsequent years, two groups of workers who examined both the chemical and enzymic oxidation of DHMA established that the reaction indeed goes through the conventional quinone intermediate (Scheme 1). Ortiz *et al.* (1988) proposed that mandeloquinone causes oxidative decarboxylation of DHMA to DHBald. However, Land and his associates (Bouheroum *et al.*, 1989), on the basis of pulse-radiolysis studies, discounted this route and favoured a unimolecular decarboxylation of mandeloquinone to the quinone methide intermediate and its subsequent tautomerization to DHBald. But until now no-one has provided any evidence for the formation of the quinone methide intermediate.

Quinone methides in general are quite unstable and tend to undergo rapid Michael 1,6-addition reactions with available nucleophiles to regenerate the benzenoid ring (Sugumaran, 1988). However, α -hydroxy-substituted quinone methides do not participate in such reactions owing to their tendency to undergo intramolecular rearrangement. Both dieneone-phenol rearrangement and keto-enol tautomerization drive these molecules to form oxo-substituted catechols. We have witnessed this type of reaction in the case of quinone methides derived from *N*- β -alanyl noradrenaline (Saul & Sugumaran, 1990),

N-acetylnoradrenaline (Saul & Sugumaran, 1990), 3,4-dihydroxyphenylglycol (Saul & Sugumaran, 1990) and 3,4-dihydroxybenzyl alcohol (Sugumaran *et al.*, 1991). Therefore quinone methides derived from the decarboxylation of mandeloquinones must also be highly unstable and exhibit a similar reaction. Hence they cannot be readily detected or isolated. To characterize the quinone methide intermediate, we reasoned that, if we substitute the α -proton of DHMA with a methyl group, then the enzymic oxidation of the resultant DHPLA should generate DHAP (see Scheme 1, R = CH₃). Therefore we chemically synthesized this new compound and tested it as a substrate for mushroom tyrosinase. The results shown in Fig. 1 clearly indicate that the course of oxidation of DHPLA is similar to that of DHMA. Accordingly, we could identify DHAP as the stable product of the reaction in near-quantitative yield, thereby indicating indirectly that quinone methide is a transient intermediate of the reaction.

The tyrosinase-catalysed oxidative decarboxylation of DHMA has been shown to proceed via the conventional quinone intermediate (Ortiz *et al.*, 1988; Cabanes *et al.*, 1988; Bouheroum *et al.*, 1989). In the present study, evidence could also be obtained for the transient formation of DHPLA quinone in the reaction mixture (Fig. 2). The remarkable instability and unimolecular decarboxylation of mandeloquinones to quinone methides is quite intriguing. Although the related carboxymethyl-*o*-benzoquinone is also quite unstable, it does not exhibit a similar decarboxylation (Sugumaran *et al.*, 1989b).

The stability of quinones varies depending on the conditions. Dopaquinone and related compounds, owing to the presence of an appropriately positioned internal amino group (nucleophile), rapidly undergo intramolecular Michael 1,4-nucleophilic addition (and hence cyclization) reactions (Prota, 1988). Similarly, both carboxymethyl-*o*-benzoquinone and carboxyethyl-*o*-benzoquinone exhibit intramolecular cyclization reactions even though the carboxy group is a very weak nucleophile (Sugumaran *et al.*, 1989a,b). However, in the case of mandeloquinone we have not observed such a reaction (Sugumaran, 1986, and the present study).

Isomerization of quinones to quinone methides is yet another frequently observed reaction. Thus, Witkop and his associates proposed the formation of quinone methides from the quinone of dopamine derivatives as a route to the production of noradrenaline derivatives (Senoh & Witkop, 1959). Although this reaction has been shown to be accomplished by a direct enzyme-catalysed hydroxylation route (Kaufman *et al.*, 1962), we have since demonstrated the occurrence of this reaction for the production of *N*-acetylnoradrenaline derivatives in insect cuticle (Saul & Sugumaran, 1988, 1990). During our studies we also confirmed the facile tautomerization of a number of quinones

to unstable quinone methides (Sugumaran, 1990). Thus quinone methide formation could account for (a) conversion of 3,4-dihydroxyphenylacetic acid into DHMA (Sugumaran *et al.*, 1989b), (b) oxidative deamination of 3,4-dihydroxybenzylamine (Sugumaran, 1990), (c) oxidative conversion of 3,4-dihydroxybenzyl alcohol into DHBALD (Sugumaran *et al.*, 1991), (d) transformation of dihydrocaffeic acid derivatives into caffeic acid derivatives (Sugumaran *et al.*, 1989a,c), (e) oxidative dimerization of 1,2-dehydro-*N*-acetyldopamine (Sugumaran *et al.*, 1990b) and (f) enzymic oxidation of α -methyl-dopa methyl ester (Sugumaran *et al.*, 1990a). But so far the novel decarboxylation of quinones to quinone methides seems to be a unique reaction characteristic of only mandeloquinones.

This work was supported in part by grants from University of Massachusetts at Boston (BRSG, Ed. Needs, Fac. Dev., and Healey) and National Institutes of Health (RO1-AI-14753). We thank Professor J. P. Anselme for the use of his n.m.r. and i.r. spectral facilities.

REFERENCES

- Bouheroum, M., Bruce, J. M. & Land, E. J. (1989) *Biochim. Biophys. Acta* **998**, 57–62
- Cabanes, J., Sanchez-Ferrer, A., Bru, R. & Garcia-Carmona, F. (1988) *Biochem. J.* **256**, 681–684
- Kaufman, S., Bridgers, W. F., Eisenberg, F. & Friedman, S. (1962) *Biochem. Biophys. Res. Commun.* **9**, 497–503
- Lerch, K. (1983) *Mol. Cell. Biochem.* **52**, 125–138
- Ortiz, F. M., Serrano, J. T., Lopez, J. N. R., Castellanos, R. V., Feruel, J. A. L. & Garcia-Canovas, F. (1988) *Biochim. Biophys. Acta* **957**, 158–163
- Prota, G. (1988) *Med. Res. Rev.* **8**, 525–556
- Robb, D. A. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., ed.), vol. 2, pp. 207–240, CRC Press, Boca Raton
- Saul, S. J. & Sugumaran, M. (1988) *FEBS Lett.* **237**, 155–158
- Saul, S. J. & Sugumaran, M. (1990) *J. Biol. Chem.* **265**, 16992–16999
- Senoh, S. & Witkop, B. (1959) *J. Am. Chem. Soc.* **81**, 6222–6231
- Sugumaran, M. (1986) *Biochemistry* **25**, 4489–4492
- Sugumaran, M. (1988) *Adv. Insect Physiol.* **21**, 179–231
- Sugumaran, M. (1990) in *Biological Oxidation Systems* (Reddy, C. C., Hamilton, G. A. & Madyastha, K. M., eds.), vol. 1, pp. 347–363, Academic Press, New York
- Sugumaran, M., Dali, H., Kundzicz, H. & Semensi, V. (1989a) *Bioorg. Chem.* **17**, 443–453
- Sugumaran, M., Semensi, V., Dali, H. & Mitchell, W. (1989b) *Bioorg. Chem.* **17**, 86–95
- Sugumaran, M., Semensi, V., Dali, H. & Saul, S. J. (1989c) *FEBS Lett.* **255**, 345–349
- Sugumaran, M., Dali, H. & Semensi, V. (1990a) *Bioorg. Chem.* **18**, 144–153
- Sugumaran, M., Schinkmann, K. & Dali, H. (1990b) *Arch. Insect Biochem. Physiol.* **14**, 93–109
- Sugumaran, M., Semensi, V., Dali, H. & Nellaippan, K. (1991) *Arch. Insect Biochem. Physiol.* **16**, 31–44