Aggregation equilibria of tyrosinases of Harding–Passey mouse melanoma

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1. The purification of two isoenzymes of tyrosinase has been carried out in Harding–Passey mouse melanoma. One is found in the cytosol and the other one bound to melanosomes. Both migrate as single bands on sodium dodecyl sulphate/polyacrylamide gels, having an apparent M_r of 58000. 2. Solubilized particulate tyrosinase showed an aggregation equilibrium involving a monomer, tetramer, octamer and a high- M_r micellar form with Brij 35, the solubilizing agent. H.p.l.c. studies indicated a interconversion between those species, the monomer contribution increasing with the sample dilution. The tetramer and the octamer probably represent the predominant forms *in vivo*. 3. Soluble tyrosinase showed a simpler aggregation equilibrium, involving two forms, monomer and tetramer, with the same interconversion pattern. 4. Fluorescence studies suggested that tryptophan residues were exposed to the aqueous environment when tyrosinase was dissociated by dilution. Tyrosinase shows a tendency to aggregate, at low protein concentration, and a resistance to dissociation by urea or SDS so remarkable that gel-permeation chromatography in 4M-urea does not affect the equilibrium, and the band obtained on SDS/polyacrylamide-gel electrophoresis is a dimer.

Tyrosinase (EC 1.14.18.1) is the key enzyme in melanogenesis. It catalyses the conversion of tyrosine into dopa (cresolase activity) and, in turn, to dopaquinone (catecholase activity). Purification and structural studies of tyrosinase have been conducted from bacteria, plants and animal sources. Unlike bacteria and plants, which contain only soluble tyrosinases, mammalian tissues are known to contain tyrosinases in both soluble and insoluble particulate forms, since Brown & Ward (1958) reported the separation of multiple forms of mouse melanoma tyrosinase. Most of the tyrosinase activity from that source is in particulate form (Burnett, 1971), bound to the melanosome (melanin-producing organelle), but the activity of the soluble form changes in response to several factors (Chen & Singh, 1974; Hearing *et al.*, 1978). Although different soluble isoenzymes of tyrosinase have been described by several authors (Burnett, 1971; Hearing *et al.*, 1978, 1981; Ohtaki & Miyazaki 1973), it seems that the enzyme occurs predominately in a glycosylated form, and in

predominately in a glycosylated form, and interconversion of the different enzyme forms can be demonstrated (Nishioka, 1977, 1978), the other isoenzymes being either the form of the tyrosinase molecule *de novo* (Hearing *et al.*, 1981) or products of partial degradation (Tomita *et al.*, 1983). The M_r values reported for the tyrosinase isoenzymes are in the range 55000–75000 (Burnett, 1971; Nishioka, 1978; Hearing *et al.*, 1981), but a value of 102000 was reported by Iwata & Takeuchi (1977) for deoxycholate-solubilized particulate tyrosinase from B-16 mouse melanoma. However, a study of the native oligomeric structure of melanoma tyrosinase has not been carried out, in spite of the early reports by Burnett *et al.* (1967), suggesting that tyrosinases exist in various molecular forms within the living cell, and the self-association equilibria described for other non-mammalian tyrosinases (Jolley *et al.*, 1969; Peñañiel *et al.*, 1982).

The present paper describes the existence of aggregation equilibria for both particulate and soluble forms of Harding–Passey-mouse-melanoma tyrosinase, showing that the interactions between the subunits are so strong that some species are resistant to dissociation by urea or SDS (Burnett *et al.*, 1967; Burnett, 1971).

Abbreviations used: dopa, 3,4-dihydroxyphenylalanine; SDS, sodium dodecyl sulphate.

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Experimental

Chemicals

DEAE-Sephadex A-50 for ion-exchange chromatography and Sephadex G-200 for gel-filtration chromatography were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Acrylamide and bisacrylamide were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Lysozyme, trypsinogen, pepsin, ovalbumin, bovine serum albumin, β -galactosidase, catalase and L-dopa were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Sucrose and urea were purchased from Merck (Darmstadt, Germany). Cytochrome *c*, aldolase and ferritin were from Boehringer-Mannheim (Mannheim, Germany). All the other reagents used were analytical grade from local suppliers.

Melanomas

Harding-Passey melanomas were obtained from the Institute of Cancer Research, Royal Cancer Hospital, London, SW7 3NU, U.K. They were propagated and maintained *in vivo* by serial transfers of dissociated tumour cells in C57/BL male mice as described by Geran *et al.* (1972). Briefly, a tumour of about 1 cm diameter was excised from the tumour-bearing animal under aseptic conditions after the mouse had been killed by cervical dislocation. The tumour was cleaned of adipose and connective tissues, weighed, and homogenized with 10 vol. (v/w) of 0.9% NaCl in a Potter homogenizer. Tumours were induced in mice by subcutaneous injection of approx. 10^5 viable cells into male mice (4–8 weeks old, 15–20 g body wt.). The animals were housed in stainless-steel cages, fed *ad libitum* and maintained in a windowless animal room with light in alternate periods of 8 h and thermostatically controlled at 21°C. Tumours started to appear within a week.

Extraction of tyrosinase

Tyrosinase isoenzymes were extracted from tumours by the slightly modified procedure of Jimbow *et al.* (1981). All procedures were performed at 4°C. Tumours were washed several times with 0.25 M-sucrose in 10 mM-phosphate buffer, pH 6.8, until the wash liquid was colourless. Then tumours were homogenized with 2 vol. (v/w) of the same buffer in a Polytron homogenizer. The homogenate was centrifuged at 700 g (r_{av} , 8.1 cm) for 20 min and the supernatant was centrifuged at 105 000 g (r_{av} , 6.3 cm) for 60 min. The supernatant was passed through sterile gauze, frozen and considered as crude soluble tyrosinase. The 105 000 g precipitate was washed twice by resuspension in the same buffer and re-centrifuged. The resulting precipitate was resuspended with 0.5 vol. (v/w) of 10 mM-phosphate buffer, pH 6.8,

and solubilized by incubation for 2 h at 4°C with 1.5% Brij 35 in the same buffer. Finally, the suspension was again centrifuged at 105 000 g (r_{av} , 6.3 cm) for 60 min and the supernatant was considered as crude particulate tyrosinase.

Purification of melanoma tyrosinases

Soluble and particulate tyrosinases were purified by using a similar procedure, but, although not indicated, all buffers used in the procedure contained 0.1% Brij 35 in the case of particulate tyrosinase. The crude extracts were dialysed at 4°C against 20 vol. of 10 mM-phosphate buffer, pH 6.8. Four changes of buffer were performed during the dialysis. DEAE-Sephadex A-50 chromatography was performed in a column (2.5 cm $\times$ 80 cm) that had been previously equilibrated with 1 mM-phosphate buffer, pH 6.8. The column was washed with the same buffer (4 vol. with respect to the volume of extract applied to the column) and then the enzyme was eluted either with a linear gradient of KNO₃ (0–1 M) in 10 mM-phosphate buffer, pH 6.8 (in the case of purification of particulate tyrosinase), or with a linear gradient of MgSO₄ (0–0.25 M) in the same buffer (in the case of soluble tyrosinase), since this isoenzyme is inactivated at high salt concentration. Active fractions (5 ml) were pooled and concentrated by ultrafiltration.

Sephadex G-200 chromatography was performed in a column (2.6 cm $\times$ 65 cm) that had been equilibrated with 10 mM-phosphate buffer, pH 6.8. A 5 ml sample was applied to the column (protein concentration 1–3 mg/ml). Elution was performed with the same buffer, with a flow rate of 0.25 ml/min. Fractions (5 ml each) were collected, and the three fractions with the highest specific activities were pooled and concentrated as described above. These preparations were used for M_r determination, SDS/polyacrylamide-gel-electrophoretic purity studies, h.p.l.c. and fluorescence properties.

Enzyme activity determination

Tyrosinase was assayed essentially as described by Mason (1948). Dopa oxidation was measured spectrophotometrically at 475 nm. The reaction mixture consisted of 3.8 mM-L-dopa, 25 mM-phosphate buffer, pH 6.8, and enzyme solution, in a final volume of 1.1 ml. One enzyme unit was defined as the amount of enzyme transforming 1 μ mol of L-dopa/min at 25°C, under the above conditions. ϵ_{475} for the conversion of L-dopa into dopachrome was taken as 3800 M⁻¹·cm⁻¹ (Mason, 1948).

Protein determination

The protein content of the preparations was determined by a modified Lowry method (Hartree,

1972), with bovine serum albumin as standard. During the purification procedure, A_{280} was sometimes taken as an index of protein concentration.

Gel electrophoresis

Electrophoresis in SDS/polyacrylamide gels was performed with the discontinuous system described by Laemmli (1970). Proteins were stained as described by Fairbanks *et al.* (1971), with 0.25% Coomassie Blue R-250, and the gels scanned in a Transdyne General Corporation RFT II densitometer at 550 nm.

Non-dissociating electrophoresis was performed by the method of Weber & Osborn (1969) in 7.5% (w/v) polyacrylamide gels. Determination of dopa oxidase activity on the gels was achieved by incubating the gels for 3–4 h in 25 mM-phosphate buffer, pH 6.8, containing 0.75 mg of L-dopa/ml. Densitometry was carried out by scanning the gels at 475 nm in the densitometer described above.

Centrifugation in sucrose gradients

The determination of $s_{20,w}$ for tyrosinase was performed as described by McEwen (1967). Centrifugation was carried out at 55000 rev./min (r_{av} 9.2 cm) for 22 h in a SW60 rotor with a L8-70 Beckman ultracentrifuge. Cellulose nitrate tubes (11 mm $\times$ 60 mm) were filled by a Beckman gradient former with a linear gradient of sucrose from 24 to 58 g/100 ml in 10 mM-phosphate buffer, pH 6.8; 200 μ l of tyrosinase samples or standard proteins were applied to the top of the tubes.

H.p.l.c. studies

The purified soluble and particulate tyrosinase isoenzymes were applied to a gel-permeation column (Protein PAK 300 SW; 0.75 cm $\times$ 30 cm) with Waters h.p.l.c. equipment. The column was equilibrated with 50 mM-phosphate buffer, pH 6.5, containing, only in the case of particulate tyrosinase, 0.1% Brij 35. The work pressure was 30.4–40.5 MPa (30–40 atm) and the flow 0.9 ml/min. The volume of samples applied ranged from 25 μ l (concentrated samples) to 100 μ l (diluted samples). Protein peaks were detected by the A_{280} and integrated with a 3392A Hewlett-Packard integrator.

Fluorescence studies

Fluorescence measurements were performed with a MPF-44B Perkin-Elmer spectrofluorimeter equipped with an Hitachi DCSU-2 differential unit for correction of spectra and polarization studies. Spectra were corrected with respect to a 0.05% Rhodamine B solution in glycerol. All samples were previously filtered through a 0.45 μ m-pore-size Millipore filter. Fluorescence-

polarization measurements were performed with Polaroid polarizers placed between the excitation beam and the sample and between the sample and the emission photomultiplier, forming a 90°C angle. Once the intensity of horizontal and vertical polarized light was measured, the polarization index was automatically calculated by the DCSU-2 unit.

Results and discussion

Purification process

Fig. 1 shows the elution profile of particulate tyrosinase in DEAE-Sephadex A-50. The enzyme was eluted with a linear gradient of KNO_3 , since sodium halides are inhibitory towards tyrosinase (Peñafiel *et al.*, 1984). The recovered activity was 80% as a single peak (0.6 M- KNO_3) and the purification was 5-fold. Active fractions were collected and concentrated by ultrafiltration to 10 ml; 5 ml of this preparation was subjected to Sephadex G-200 chromatography and the elution profile is shown in Fig. 2. It can be seen that two peaks of activity were obtained. The first one was eluted at the void volume of the column, indicating a high M_r . To investigate the dissociation of that species, the Sephadex G-200 column was equilibrated in the presence of 4 M-urea in the same buffer, and again 5 ml of tyrosinase partially purified with DEAE-Sephadex was applied to the column. The elution profile was closely similar to that obtained in Fig. 2, indicating the stability of the aggregate, which is highly resistant to dissociation by urea. This stability has already been described for tyrosinases from other sources (Burnett *et al.*, 1967; Peñafiel *et al.*, 1982).

The second peak of tyrosinase activity on Sephadex G-200 chromatography was concentrated by ultrafiltration, and it was purified 57-fold

Fig. 1. Elution profile of particulate tyrosinase chromatographed on DEAE-Sephadex A-50
●, A_{280} ; ○, enzyme activity; —, $[KNO_3]$.

The preparation with this grade of purification was used in h.p.l.c. and fluorescence experiments. Electrophoresis on 7.5%-(w/v)-polyacrylamide gels of these particulate tyrosinase samples containing 25 μ g of protein showed essentially a single band when stained with Coomassie Blue R-250, indicating a purity greater than 90%.

Soluble tyrosinase was purified by a similar procedure. However, it is noteworthy that the initial dialysis of the crude extract caused a marked activation of tyrosinase activity, probably due to the elimination of diffusible inhibitors present in the cytosolic fraction, as described in Fortner melanoma (Sato & Mishima, 1967) and other sources (Madhosingh, 1975). The dialysed soluble tyrosinase was applied to the DEAE-Sephadex A-50 chromatography. The elution (profile not shown) was achieved with a linear gradient of MgSO_4 (0–0.25 M), because higher concentrations of univalent-metal salts produced an inhibition of enzyme activity. The peak eluted at 0.12–0.14 M- MgSO_4 was collected, concentrated, and subjected to Sephadex G-200 chromatography. A single peak of tyrosinase activity was obtained in this case, and no activity was detected at the void volume of the column. Table 1 shows the steps in the purification procedure and the specific activity of the enzyme at each stage. Electrophoresis in 7.5%-polyacryl-

amide gels of these samples containing even 50 μ g of protein showed a single band when stained with Coomassie Blue R-250, also indicating a purity of more than 90%.

Several authors have reported the existence of two (or more) soluble isoenzymes (Burnett *et al.*, 1967; Burnett, 1971; Hearing *et al.*, 1978, 1981; Ohtaki & Miyazaki, 1973; Miyazaki & Ohtaki, 1975). However, all attempts in our laboratory to detect more than one soluble isoenzyme were unsuccessful, so samples of the dialysed or purified extract were subjected to 7.5%-polyacrylamide-gel electrophoresis in the absence of SDS and the gels specifically stained for dopa oxidase activity as described in the Experimental section, showing a single band with the same mobility for both samples.

Some explanations for these results are as follows. (a) The isoenzymes show identical immunological and kinetic features (Ohtaki & Miyazaki, 1973) and any differences would seem to be attributable exclusively to the extent of glycosylation of the polypeptide chains. (b) The relative concentration of each isoenzyme is dependent on the tumour (Burnett *et al.*, 1967). (c) The number of isoenzymes changes during the tumour growth in B-16 melanoma (Chen & Singh, 1974). (d) Artefacts produced by electrophoretic desamidation (Hearing *et al.*, 1978, 1981) would also contribute to the diversity of cytosolic isoenzymes reported in the literature. (e) Finally, Tomita *et al.* (1983) recently claimed that soluble isoenzymes may be partially degraded species produced at necrotic regions of the tumour from the melanosome-bound tyrosinase, the only form of the enzyme to play a role in the cell.

M_r determination

The subunit composition of soluble and particulate isoenzymes of tyrosinase was studied first in 7.5%-polyacrylamide gels containing SDS. Both isoenzymes showed the same mobility, corresponding to species of M_r 58000 $\pm$ 1000, and are in agreement with other reported values (Burnett, 1971; Hearing *et al.*, 1981; Tomita *et al.*, 1983).

However, the M_r of native tyrosinases determined by gel chromatography in Sephadex G-200

Fig. 2. Elution profile of particulate tyrosinase chromatographed on Sephadex G-200
●, A_{280} ; ○, enzyme activity.

Table 1. Purification of soluble tyrosinase

Step	Total protein (mg)	Specific activity (munits/mg)	Purification factor (fold)	Yield (%)
Cytosolic supernatant	1399	0.47	1	100
Dialysis residue	1161	1.43	3.1	249.2
DEAE-Sephadex	238.7	5.50	11.7	196.8
Gel permeation on Sephadex G-200	46.4	23.2	49.4	161.3

was approx. $133\,500 \pm 1500$ for the soluble form and $137\,000 \pm 1000$ for the retained peak of the particulate form (mean $\pm$ S.E.M., $n=3$). These results showed that the native enzyme is mainly found as an oligomeric structure with an M_r higher than that obtained under the dissociating conditions used for gel electrophoresis. The Stokes radii (Ackers, 1970) for soluble and particulate isoenzymes were 4.49 ± 0.02 and 4.52 ± 0.01 nm respectively.

M_r values of native tyrosinases were also studied by sucrose-gradient centrifugation. The migration profiles of the different forms of tyrosinase are shown in Fig. 3. First, it can be seen that the peak eluted at the void volume in gel-permeation chromatography of particulate tyrosinase did not migrate in the sucrose gradient as much as the retained peak or the soluble tyrosinase did, in spite

of the larger molecular size of that form. This behaviour can be only explained by assuming that the aggregate of large molecular size is a micelle formed by enzyme and detergent molecules, with lower density than the 'free' particulate and soluble forms of tyrosinase. Secondly, the shoulders in the migration profile (Figs. 3a and 3b) may be attributed to an equilibrium between the micellar and the free forms of particulate tyrosinase. Thirdly, the sedimentation constants for the particulate and soluble native tyrosinases were 7.09 and 7.84S, corresponding to M_r values of 134000 and 147000 respectively (Fig. 3c, insert), in agreement with the values obtained by gel-filtration chromatography.

H.p.l.c. studies

The data obtained strongly suggested that melanoma tyrosinase would occur as a oligomeric species composed of two subunits of M_r approx. 58000. In turn, particulate tyrosinase would be in equilibrium between a micellar (with Brij 35) and a free form. An association-dissociation equilibrium has been described for tyrosinases from other sources (Vanneste & Zuberbühler, 1974; Iborra *et al.*, 1981), and an h.p.l.c. study was performed to clarify the significance of the different melanoma tyrosinases obtained so far. When a sample of purified particulate tyrosinase (0.46 mg of protein/ml) was submitted to h.p.l.c., four peaks were resolved, as shown in Fig. 4(a). Moreover, when the 5.92, 7.55 and 8.60 min peaks were collected and resubmitted to h.p.l.c., an interconversion between them was observed (Figs. 4b, 4c and 4d), always increasing the fourth one, whereas that peak re-applied to the column yielded the same retention time again (11.92 min). Once the column was calibrated with standard proteins, the corresponding M_r under these conditions for 5.92, 7.55, 8.60 and 11.92 min retention time were $> 500\,000$ (void volume), 355000, 182000 and 28000 respectively.

The contribution of these species to equilibrium as a function of protein dilution is shown in Fig. 5. It can be seen that the intermediate forms (M_r 182000 and 355000) decreased with dilution, the micellar form increased up to a maximum at about 100-fold dilution, and the monomeric form constantly increased to be the main form at dilutions above 10000-fold. That monomer was first detected by Burnett (1971) in melanoma tyrosinase, and it had an M_r of 30000–32000. However, that monomer has not been well characterized in subsequent work, owing to the extremely low protein concentrations (high dilutions) at which it occurs. A tetramer and other more complex species could be detected by h.p.l.c. as the main forms in which native tyrosinases occur. The

Fig. 3. Sedimentation profile of tyrosinases in sucrose density gradients

(a) Micellar form of particulate tyrosinase; (b) free form of particulate tyrosinase; (c) soluble tyrosinase. The inset shows M_r determination of particulate (P) and soluble (S) tyrosinases by sucrose-density-gradient centrifugation: 1, cytochrome c; 2, bovine serum albumin; 3, aldolase; 4, catalase.

Fig. 4. Elution profiles on h.p.l.c. of purified particulate tyrosinase, showing the interconversion between the different forms

(a) H.p.l.c. chromatogram of a sample of the second peak obtained on Sephadex G-200 chromatography; (b) chromatogram of the 5.92 min-retention-time peak (in a) resubmitted to the column; (c) chromatogram of the 7.55 min-retention-time peak (in a) resubmitted to the column; (d) chromatogram of the 8.60 min-retention-time peak (in a) resubmitted to the column.

M_r values obtained by this technique are slightly different from those obtained by gel permeation or sucrose-gradient centrifugation, probably owing to the glycoprotein nature of the tyrosinase, which causes deviations in the estimation of M_r by several techniques.

Purified soluble tyrosinase also showed proof of the existence of an association-dissociation equilibrium. However, the number of species detected was lower than that observed for particulate tyrosinase. Only two forms were detected, corresponding to M_r values of 28000 and 160000. Protein dilution produced a continuous decrease in the concentration of the high- M_r form and a

Fig. 5. Contribution of the different forms of purified particulate tyrosinase in equilibrium as a function of protein concentration

The initial protein concentration was 0.46 mg/ml. ■, Monomeric form; ●, tetrameric and octameric form; ○, micellar form.

Fig. 6. Fluorescence polarization of purified particulate tyrosinase as a function of protein concentration. The initial protein concentration was 0.46 mg/ml.

concomitant increase in the concentration of the 28000- M_r species. The micellar form was not observed at all, probably due to the absence of detergent in these preparations and to the lower hydrophobicity of the soluble isoenzyme.

Fluorescence studies

Fig. 6 shows the evolution of the intrinsic fluorescence polarization of the purified particulate tyrosinase with the sample dilution. Light excitation was performed at 300 nm, and the

Fig. 7. Fluorescence spectra of purified particulate tyrosinase

----, Protein concn. 0.46 mg/ml; — protein concn. 0.23 µg/ml. The excitation wavelength was 300 nm.

fluorescence polarization was measured at 350 nm. A decrease in the intensity of polarization with the enzyme dilution, over a range where the species that mainly increases is that of M_r 500 000, can be observed (Fig. 6). This fact indicates a disordered state and a free movement of the polypeptide chains, a situation compatible with a micellar structure formed by detergent molecules and enzyme.

Fig. 7 shows the fluorescence spectra of two purified samples of protein concentration 0.46 mg/ml and 0.23 µg/ml, when respectively either oligomeric or monomeric forms predominate. Light excitation was performed at 300 nm and the results, analysed in terms of either fluorescence intensity or the wavelength of maximum emission, indicated the occurrence of a conformational change in the transition from the oligomeric to the monomeric structure. The fluorescence peak was shifted from 324 to 337 nm, and a shoulder at 350 nm appeared, strongly suggesting the exposure of tryptophan residues from the interior of the protein to a more polar aqueous environment (Teale, 1959). When the structure was dissociated by dilution, these resi-

dues would be exposed to the aqueous environment, leading to the fluorescence changes described above.

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References

- Ackers, G. K. (1970) *Adv. Protein Chem.* **24**, 343–446
- Brown, F. C. & Ward, D. N. (1958) *J. Biol. Chem.* **233**, 77–80
- Burnett, J. B. (1971) *J. Biol. Chem.* **246**, 3079–3091
- Burnett, J. B., Seiler, H. & Brown, I. V. (1967) *Cancer Res.* **27**, 880–889
- Chen, Y. M. & Singh, D. V. (1974) *Oncology* **30**, 386–389
- Fairbanks, G., Steck, T. L. & Wallach, D. F. (1971) *Biochemistry* **10**, 2606–2617
- Geran, R. I., Greenberg, N. H., MacDonald, M. M., Schumacher, A. M. & Abbott, B. J. (1972) *Cancer Chemother. Rep. Part 3* **3**, 1–103
- Hartree, E. (1972) *Anal. Biochem.* **48**, 422–427
- Hearing, V. J., Nicholson, J. M., Montague, P. M., Ekel, T. M. & Tomecki, K. J. (1978) *Biochim. Biophys. Acta* **522**, 327–339
- Hearing, V. J., Ekel, T. M. & Montague, P. M. (1981) *Int. J. Biochem.* **13**, 99–103
- Iborra, J. L., Ferragut, J. A. & Lozano, J. A. (1981) *Biochem. J.* **197**, 581–589
- Iwata, K. & Takeuchi, T. (1977) *J. Invest. Dermatol.* **68**, 88–92
- Jimbow, H., Jimbow, M. & Chiba, M. (1981) *J. Invest. Dermatol.* **77**, 213–218
- Jolley, R. L., Robb, D. A. & Mason, H. S. (1969) *J. Biol. Chem.* **244**, 1593–1599
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–682
- Madhosingh, C. (1975) *Can. J. Microbiol.* **21**, 2108–2111
- Mason, H. S. (1948) *J. Biol. Chem.* **172**, 83–99
- McEwen, C. R. (1967) *Anal. Biochem.* **20**, 114–119
- Miyazaki, K. & Ohtaki, N. (1975) *Arch. Dermatol. Forsch.* **252**, 211–216
- Nishioka, K. (1977) *FEBS Lett.* **80**, 225–228
- Nishioka, K. (1978) *Eur. J. Biochem.* **85**, 137–146
- Ohtaki, N. & Miyazaki, K. (1973) *J. Invest. Dermatol.* **61**, 339–343.
- Peñafiel, R., Galindo, J. D., Pedreño, E. & Lozano, J. A. (1982) *Biochem. J.* **205**, 397–404
- Peñafiel, R., Galindo, J. D., Solano, F., Pedreño, E., Iborra, J. L. & Lozano, J. A. (1984) *Biochim. Biophys. Acta* **788**, 327–332
- Sato, G. J. & Mishima, Y. (1967) *J. Invest. Dermatol.* **48**, 301–303
- Teale, F. W. J. (1959) *Biochemistry* **76**, 381–388
- Tomita, Y., Harin, A., Kato, C. & Seiji, M. (1983) *Arch. Biochem. Biophys.* **225**, 75–85
- Vanneste, W. H. & Zuberbühler, A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 371–404, Academic Press, New York
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412