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Loss of Haem from Cytochrome *P*-450 caused by Lipid Peroxidation and 2-Allyl-2-isopropylacetamide

AN ABNORMAL PATHWAY NOT INVOLVING PRODUCTION OF CARBON MONOXIDE

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1. Microsomal preparations undergoing lipid peroxidation produce CO and lose haem from cytochrome *P*-450. 2. The amount of CO produced does not correlate with the amount of haem lost and, after pre-labelling of microsomal haem in its bridges with 5-amino[5-^{14}C]laevulinate, the radioactivity lost from haem is not recovered as CO. 3. Similarly, when pre-labelled microsomal haem is destroyed by the action of 2-allyl-2-isopropylacetamide, no radioactivity is recovered as CO. In clear contrast, on degradation of haem by the haem oxygenase system, CO is produced in an amount equimolar to the haem lost. 4. It is concluded that (*a*) the CO produced during lipid peroxidation originates from a source different from haem and (*b*) the degradations of haem caused by lipid peroxidation and 2-allyl-2-isopropylacetamide do not involve to any significant extent evolution of the methene-bridge carbon of haem as CO.

Nishibayashi *et al.* (1968) first reported the formation of small amounts of CO during the NADPH-dependent peroxidation of microsomal lipids, but the precise source of the CO produced during this process remained obscure. Microsomal preparations undergoing lipid peroxidation show a loss of haem from cytochrome *P*-450 (De Matteis & Sparks, 1973; Levin *et al.*, 1973; Schacter *et al.*, 1973) and the possibility was therefore considered that the haem of this haemoprotein could, by peroxidative cleavage of one of its methene bridges, produce CO (Estabrook *et al.*, 1971; De Matteis & Sparks, 1973; Levin *et al.*, 1973; Schacter *et al.*, 1973). This view appeared to be supported by the observation that haem undergoing degradation both *in vivo* and *in vitro* in several other systems produces CO (reviewed by O'Carra, 1975) and also by the report that under conditions of iron-dependent lipid peroxidation the amount of CO produced was apparently very nearly equimolar to the haem lost (Schacter *et al.*, 1973).

The present findings indicate on the contrary that the CO produced during the process of lipid peroxidation does not originate from the methene bridges of haem: the amount of CO produced does not correlate with the amount of haem lost; and, when the haem of the microsomal fraction is pre-labelled in its bridges biosynthetically from the specific precursor 5-amino[5-^{14}C]laevulinate, the radioactivity lost from haem is not recovered as CO. Similar findings are presented for the 2-allyl-2-isopropylacetamide-dependent haem degradation, which has been reported to involve conversion of haem into certain green pigments (De Matteis, 1971; McDonagh *et al.*, 1976; De Matteis & Unseld, 1976). In clear contrast, when haem is degraded to bile pigments by the liver haem oxygenase system, CO is produced in an amount equimolar to the haem degraded, in agreement with findings reported both *in vivo* (Sjöstrand, 1970; Landaw *et al.*, 1970) and *in vitro* (White, 1970).

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Materials and Methods

Materials

Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NAD^+, $NADP^+$ and NADPH were obtained from Boehringer Corp. (London) Ltd., London W5 2PZ, U.K. Insta-gel was obtained from Packard Instruments Ltd., Caversham, Berks. RG4 7AA, U.K. Hopcalite (a mixture of MnO_2 and CuO, approx. 60:40, w/w) was from Hopkin and Williams, Romford RM1 1HA, U.K. 5-Amino-[5-^{14}C]laevulinic acid (49mCi/mmol) and [U-^{14}C]-linoleic acid (810mCi/mmol) were obtained from New England Nuclear G.m.b.H., Dreieichenhain, West Germany. ^{14}CO (40mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., trapped into an excess of haemoglobin from horse erythrocyte lysate in the absence of air and transferred into a gas-tight flask and stored at 4°C under N_2: no appreciable loss of radioactivity was observed over a period of 2 years.

Treatment of animals and preparation of liver fractions

Male albino rats (160–180g) of the Porton strain were starved overnight, before being killed by

decapitation. The concentration of cytochrome *P*-450 in the liver microsomal fraction was increased by giving rats two intraperitoneal injections of phenobarbitone (sodium salt), each of 80mg/kg body wt., the first 48h and the second 24h before killing. $CoCl_2,6H_2O$ was given as a single subcutaneous injection at a dose of 60mg/kg body wt., 16h before killing, to stimulate the activity of haem oxygenase (Maines & Kappas, 1974). The rats were killed and the livers perfused *in situ* with ice-cold 0.9% NaCl through the inferior vena cava. The livers were removed and homogenized (10%, w/v) in 0.25M-sucrose. To obtain a post-mitochondrial supernatant the liver homogenates were centrifuged at 9000*g* for 20min and the supernatant was again centrifuged at 18000*g* for 10min. The microsomal fraction was prepared by centrifuging the supernatant of a 9000*g* (20min) centrifugation at 105000*g* for 1h and washed once with 1.15% (w/v) KCl.

Spectrophotometric determination of CO production and cytochrome P-450 loss

Washed microsomal preparations were incubated at 37°C in a 3.5ml 1cm-light-path spectrophotometer cell with a rubber stopper. The incubation mixture contained, in a total volume of 3.3ml, the following components, with final concentrations in parentheses: microsomal proteins (0.3–0.9mg/ml); glucose 6-phosphate (4.9mM); glucose 6-phosphate dehydrogenase (0.05 unit/ml); sucrose (110mM); KCl (11.2mM); $MgCl_2$ (4.8mM); sodium phosphate buffer, pH7.4 (66.7mM); $NADP^+$ (0.19mM); and, where appropriate, NAD^+ (0.20mM), freshly dissolved $FeSO_4$ (0.2mM) or EDTA (1mM). As under these conditions CO was produced endogenously during the incubation, cytochrome *P*-450 could not be measured by the usual method (Omura & Sato, 1964) of recording a difference spectrum between the reduced microsomal preparation in the reference cell and the reduced microsomal preparation saturated with CO in the sample cell. Instead a dual-wavelength spectrophotometric assay was devised (using a Perkin Elmer 356 spectrophotometer) which allowed measurements of the difference between absorbance at 450 and 490nm, by using only one cell, i.e. the stoppered cell used for the incubation. With unincubated microsomal preparation the difference between absorbance at these two wavelengths, as measured by the new dual-wavelength assay and the conventional (Omura & Sato, 1964) method, agreed within 6%. After incubation, 0.1ml of a freshly prepared anaerobic aqueous solution of sodium dithionite (approx. 50mg/ml) was added by injection through the rubber stopper, and the difference between absorbance at 450 and 490nm corresponding to the complex of reduced cytochrome *P*-450 with endogenously produced CO was recorded. Excess CO was then added from outside to determine the total cytochrome *P*-450 content, by using an absorption coefficient of $91\,mM^{-1}\cdot cm^{-1}$ for the difference between absorbance in 450 and 490nm (Omura & Sato, 1964). The total amount of CO produced was calculated from the amount of cytochrome *P*-450–endogenous CO complex, in addition to the small amount of free CO dissolved in the incubation mixture [by using the dissociation constant of 0.2μM for the CO complex of reduced cytochrome *P*-450 (Nishibayashi *et al.*, 1968)].

Isotopic experiments

The degradation of ^{14}C-labelled haem (either exogenous haemin or the endogenous haem of the microsomal fraction) and its conversion into labelled CO was investigated *in vitro* by incubating post-mitochondrial supernatant or microsomal fraction in the incubation mixture, the composition of which has been given above. The haem of the liver microsomal fraction was pre-labelled *in vivo* by injecting rats intraperitoneally with 10μCi (0.24μmol) of 5-amino-[5-^{14}C]laevulinate 2h before killing. [^{14}C]Haemin (0.1mCi/mmol) was prepared biosynthetically by incubating chicken erythrocyte lysate with 5-amino-[5-^{14}C]laevulinate as described by Dresel & Falk (1954) and isolating the labelled haemin in a crystalline form (Labbe & Nishida, 1957); it was added to the incubation mixture to a final concentration of 33.3μM, as methaemalbumin (Tenhunen *et al.*, 1968). Incubation was for 30min at 37°C with shaking (60cycles/min) in stoppered 10ml flasks for the study of haem degradation, or in 100ml flasks with a Suba-Seal rubber stopper (and containing a Teflon-coated magnetic stirrer) for the production of labelled CO, with incubation volumes of 6ml and 60ml respectively. Samples (2ml) of the incubation mixture were taken from the 10ml flasks, before and after incubation; after addition of carrier haem as horse erythrocyte lysate, haemin was crystallized (Labbe & Nishida, 1957) and the crystals were dissolved in pyridine. A small portion of the solution was taken for determination of haem as the pyridine haemochrome (Paul *et al.*, 1953); another (equivalent to 0.6–1.5mg of haemin) was dried on a strip of filter paper folded in a concertina fashion, and the haem was then converted into $^{14}CO_2$ by combustion in an Intertechnique Oxymat apparatus and its radioactivity determined by liquid-scintillation counting in toluene/methanol/2-phenylethylamine/water (40:22:33:5, by vol.), containing 1% of 2,5-diphenyloxazole.

Each sealed 100ml flask was attached to an apparatus consisting of the following connected in series: a soda-lime column, a heated (100–120°C) Hopcalite column (Lamb *et al.*, 1922), two CO_2 traps each containing ethanolamine and 2-methoxyethanol (1:2, v/v; Landaw & Winchell, 1966) and

finally a vacuum pump. After injection of 10ml of a 0.73M-$K_3Fe(CN)_6$ solution (Coburn *et al.*, 1964) into the flask to oxidize the haem iron and thereby release any CO bound to the haem of haemoproteins, CO_2-free air was drawn over the surface of the incubation mixture (mixed by the action of the magnetic stirrer) and then through the system at a rate of 1.5 litres/min; the labelled CO was converted into CO_2, trapped and its radioactivity measured by liquid-scintillation counting by adding 1 ml of ethanolamine/2-methoxyethanol trap to 10ml of Insta-gel. At the end of each run the efficiency of the system was assessed by injecting a known amount of ^{14}CO as carboxyhaemoglobin (see above) into the incubation mixture and determining the radioactivity recovered in the final traps (recovery was generally above 90%).

Results

In the first experiment, microsomal preparations from rats pre-treated with phenobarbitone were incubated in stoppered spectrophotometric cells with the various additions indicated in Table 1, and the amount of CO produced during the incubations was related to the cytochrome *P*-450 lost. CO production was dependent on conditions previously reported (Wills, 1969; Poyer & McCay, 1971; De Matteis & Sparks, 1973) to stimulate lipid peroxidation: it was increased by NADP(H) and still further when NAD^+ or $FeSO_4$ were added together with $NADP^+$, and could be markedly decreased by EDTA (see also Nishibayashi *et al.*, 1968), presumably through removal of non-haem iron, which is known to be essential for microsomal lipid peroxidation (Wills, 1969). The loss of cytochrome *P*-450 observed under these conditions did not correlate with, and was usually far smaller than, the production of CO. The discrepancy was particularly evident with an incubation time of 2min, especially when lipid peroxidation was stimulated by addition of both $NADP^+$ and $FeSO_4$; then CO production was more than 8-fold greater, in molar terms, than the loss of cytochrome *P*-450. A similar excess of CO production, when related to loss of either cytochrome *P*-450 (Table 1) or of total microsomal haem (results not shown), was found with microsomal preparations of rats that had not been pre-treated with phenobarbitone. These observations indicate clearly that the CO produced during the process of microsomal lipid peroxidation cannot originate (at least not entirely) from cleavage of the methene bridges of haem, but may be produced instead from the lipids themselves undergoing peroxidative degradation. A similar conclusion has been reached by Wolff & Bidlack (1976), who have also found that isolated phospholipids (which presumably contained no haem) produced a small amount of CO on peroxidation.

Table 1 also shows that, in the presence of EDTA, 2-allyl-2-isopropylacetamide or its metabolites caused a further loss of cytochrome *P*-450, but a decreased production of CO [as compared with those caused by NADP(H) alone], strongly suggesting that the 2-allyl-2-isopropylacetamide-dependent loss of cytochrome *P*-450 haem also does not involve cleavage of the haem methene bridges as CO.

Table 1. *Production of CO and loss of cytochrome P-450 in vitro on incubation of liver microsomal preparations*
Liver microsomal fractions from starved rats, some of which were also treated with phenobarbitone, were incubated in spectrophotometric cells with the additions and for the times indicated. Results are means ± S.E.M. of three observations, or averages (with individual observations in parentheses). The initial amounts of cytochrome *P*-450 in the spectrophotometric cells were 9.5–10.2nmol for preparations from phenobarbitone-treated rats and 3.2–3.5nmol for those from rats without such treatment. † $P < 0.01$; ‡ $P < 0.001$, when compared with corresponding values obtained with $NADP^+$ alone (Student's *t* test).

Treatment	Addition	Incubation time (min)	CO formed (nmol)	Cytochrome *P*-450 lost (nmol)
Phenobarbitone	$NADP^+$	2	1.08 ± 0.07	0.69 ± 0.09
	$NAD^+ + NADP^+$	2	1.42 ± 0.06†	0.53 ± 0.05
	$FeSO_4 + NADP^+$	2	3.26 ± 0.18‡	0.36 ± 0.22
	None	10	Nil	0.81 (0.81, 0.81)
	NAD^+	10	0.10 (0.06, 0.14)	1.01 (0.66, 1.35)
	$NADP^+$	10	2.32 (1.80, 2.83)	1.92 (1.81, 2.03)
	$NAD^+ + NADP^+$	10	4.45 (4.87, 4.03)	1.34 (0.98, 1.70)
	$FeSO_4 + NADP^+$	10	4.06 (3.84, 4.28)	1.86 (1.81, 1.92)
	EDTA	10	Nil	1.21 (1.14, 1.28)
	EDTA + $NADP^+$	10	0.15 (0.14, 0.17)	1.66 (1.50, 1.83)
	EDTA + $NADP^+$ + 2-allyl-2-isopropylacetamide	10	Nil	2.24 (2.19, 2.30)
None	None	10	Nil	0.14 (0.13, 0.15)
	$NADP^+$	10	3.27 (3.14, 3.41)	0.94 (0.89, 1.00)

Table 2. *Loss of radioactivity from haem and recovery of radioactivity from haem as CO*

Liver microsomal fractions or post-mitochondrial supernatants were incubated with the additions indicated, as described in the Materials and Methods section. The loss of radioactivity from either endogenous (Expts. 1 and 2) or exogenous (Expt. 3) haem (labelled with 5-amino[5-^{14}C]laevulinate) was measured and related to the radioactivity recovered as ^{14}CO. The latter was expressed as a percentage of the radioactivity lost from haem, on the assumption that 1 mol of CO would be expected from each mol of haem lost. The results are those of individual observations or averages with individual observations in parentheses. The sign — indicates that production of ^{14}CO was not measured.

Expt. no.	Treatment	Source of enzyme	Addition	Loss of radioactivity from haem (% of haem radioactivity of sample not incubated)	Radioactivity recovered as CO (% of radioactivity expected on a 1:1 haem/CO molar basis)
1	Rat starved for 24h	Microsomal fraction	None	5.0 (3.0, 7.0)	—
			$NADP^+$	41.0 (40.0, 42.0)	6.7 (5.2, 8.3)
			$NADP^+$ + EDTA	6.1 (6.0, 6.3)	—
			$NADP^+$ + EDTA + 2-allyl-2-isopropylacetamide	25.5 (23.0, 28.0)	6.2 (5.8, 6.7)
2	Rat starved for 24h	Post-mitochondrial supernatant	$NADP^+$ + EDTA	3.5 (1.0, 6.0)	—
			$NADP^+$ + EDTA + 2-allyl-2-isopropylacetamide	44.6 (43.2, 46.0)	4.4
3	Rat starved for 24h and given $CoCl_2$	Post-mitochondrial supernatant	$NADP^+$	36.1 (29.0, 43.3)	95.0 (97.5, 92.5)

To obtain conclusive evidence that the loss of haem observed during either the process of lipid peroxidation or the action of 2-allyl-2-isopropylacetamide does not involve evolution of CO from the methene bridges, haem was specifically labelled in its carbon bridges and the loss of radioactivity observed from haem was compared with the radioactivity recovered as CO (Table 2). NADPH could catalyse haem destruction in incubated liver microsomal preparations undergoing lipid peroxidation. This effect of NADPH could be completely abolished by EDTA. When the NADPH-dependent lipid peroxidation was inhibited by EDTA, 2-allyl-2-isopropylacetamide still caused loss of radioactivity from pre-labelled haem on incubation of either microsomal suspension or of post-mitochondrial supernatants. Finally, liver post-mitochondrial supernatants prepared from animals that had been pre-treated with $CoCl_2$ in order to stimulate their liver haem oxygenase activity (Maines & Kappas, 1974), could degrade exogenous [^{14}C]haem added as methaemalbumin.

When the haem molecule is labelled with 5-amino-[5-^{14}C]laevulinate, one-eighth of the total haem radioactivity is associated with each of its four methene bridges: therefore, during haem degradation, one would expect to recover as CO one-eighth of the radioactivity lost from haem, if 1 mol of CO was produced for each mol of haem lost. This was the case when haem was degraded by the haem oxygenase system, in agreement with previous findings (Landaw *et al.*, 1970; Sjöstrand, 1970). In contrast, in both the lipid peroxidation and 2-allyl-2-isopropylacetamide-dependent losses of haem only 6–7% of the expected yield of labelled CO was obtained (Table 2).

Discussion

The following conclusions can be drawn from the present findings. First, the CO that is produced during the process of lipid peroxidation probably originates from a source different from haem, perhaps the lipids themselves undergoing peroxidative degradation (see also Wolff & Bidlack, 1976). Brown & King (1976) studied the degradation of haem by the haem oxygenase system of spleen microsomal fraction under an atmosphere enriched in $^{18}O_2$. They found that the $C^{18}O$ produced exceeded the amount expected from the quantity of bilirubin formed. Atmospheric O_2 is utilized during the process of lipid peroxidation; therefore, if under the conditions of the experiments of Brown & King (1976) lipid peroxidation by the spleen microsomal fraction was taking place, CO produced from the lipids being peroxidized would be expected to be labelled with ^{18}O, and this might explain their finding. A possible source of CO within the lipids being peroxidized could be the unsaturated fatty acids themselves, since these undergo extensive degradation, giving rise to a

variety of degradation products, including carbonyl compounds (Poyer & McCay, 1971), which are further degraded, and certain hydrocarbons (Riely *et al.*, 1974). Direct evidence for this was sought by incubating [U-^{14}C]linoleic acid aerobically at pH7.4 for 1h either on its own or in the presence of liver microsomal preparations undergoing lipid peroxidation because of NADPH addition, or by incubating it at pH4.6 for 18h in the presence of α-linolenic acid undergoing active peroxidation because of ascorbate addition. Under similar conditions production of a small amount of thiobarbituric acid-positive material from linoleic acid has been reported (Wilbur *et al.*, 1949). No significant conversion of the label into ^{14}CO could be demonstrated. This negative finding does not exclude, however, that CO originates from other unsaturated fatty acids: arachidonic acid (Levin *et al.*, 1973) is a much more important site of peroxidative degradation (than linoleic acid) and it may therefore be the source of CO production.

It can also be concluded from the present work that the losses of haem that accompany either lipid peroxidation or the action of 2-allyl-2-isopropylacetamide do not involve to any significant extent liberation of the methene-bridge carbon of haem as CO. These processes contrast with the physiological pathway of haem degradation, where both CO and bilirubin are produced in amounts equimolar to the haem lost, and must therefore involve conversion of haem into abnormal derivatives (hereafter called green pigments). This conclusion is in keeping with the reported observation that a modified form of haem accumulates in the liver after 2-allyl-2-isopropylacetamide treatment (De Matteis & Unseld, 1976), which is responsible for the brown–green colour of the microsomal fraction and which, on treatment with acids, gives rise to modified porphyrins (Schwartz & Ikeda, 1955; McDonagh *et al.*, 1976; Unseld & De Matteis, 1976). The spectrum of the most abundant of these is similar to that of porphyrins bearing electron-withdrawing substituents in their 2- and 4-positions, i.e. the positions normally occupied by the vinyl groups of protoporphyrin. In whole-animal studies 2-allyl-2-isopropylacetamide has been reported to decrease the exhalation of ^{14}CO both from injected [^{14}C]haemin (Landaw *et al.*, 1970) and from haem labelled *in vivo* with 5-amino[5-^{14}C]-laevulinate (Guzelian & Bissell, 1976), but to increase the exhalation of ^{14}CO when [2-^{14}C]glycine was used to label *in vivo* the methene bridges of haem (Landaw *et al.*, 1970). The reason for this difference is not clear. Landaw *et al.* (1970) have also found that in rats treated with 2-allyl-2-isopropylacetamide significantly more ^{14}CO was produced than [^{14}C]bilirubin. This last observation is also difficult to interpret at present: perhaps ^{14}CO is produced *in vivo* not at the stage of formation of the green pigments but during their further metabolism.

Microsomal preparations undergoing lipid peroxidation acquire a brown–green colour (De Matteis & Sparks, 1973) reminiscent of that seen in the microsomal fraction of the liver of rats treated with 2-allyl-2-isopropylacetamide. The green pigments produced during lipid peroxidation have not yet been characterized, but in view of the present finding that there is no concomitant evolution of labelled CO from haem, the possibility exists that these pigments are similar to those produced by 2-allyl-2-isopropylacetamide. Perhaps in both processes reactive species such as peroxides or free radicals are responsible. These may modify the structure of haem by oxidative attack, for example at the β-positions of the pyrrole rings, without generating open tetrapyrroles of the bile-pigment type.

Masters & Schacter (1976) have studied the degradation of haem by the purified microsomal flavoprotein NADPH–cytochrome *c* reductase and found that, in the absence of haem oxygenase, the flavoprotein will degrade haem, but mostly to compounds that cannot serve as precursors of bilirubin. They suggest that these compounds are unphysiological isomers of biliverdin, but they could equally well be abnormal derivatives unrelated to biliverdin and similar to the green pigments considered in the present paper. It is known that NADPH–cytochrome *c* reductase can utilize oxygen as an electron acceptor to generate superoxide anion (Aust *et al.*, 1972) and that the latter can then be converted, either spontaneously or enzymically, into H_2O_2 (McCord & Fridovich, 1968). It is possible that H_2O_2 or hydroxyl radicals derived from it (Beauchamp & Fridovich, 1970) are responsible for changing haem into green pigments, for example by attacking some of the β-substituents of its pyrrole rings (e.g. the vinyl substituents), but without producing significant amounts of CO and bile pigments. Compatible with this interpretation is the finding that the degradation of haem by the flavoprotein on its own was markedly inhibited by catalase (Masters & Schacter, 1976), whereas that observed when haem oxygenase was also present was not (Kikuchi & Yoshida, 1976). A study of the evolution of ^{14}CO from labelled haem incubated with the flavoprotein (along the lines of the present work) should clarify whether under the experimental conditions of Masters & Schacter (1976) haem is converted into biliverdins or into compounds similar to the green pigments that have been considered in the present paper.

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References

Aust, S. D., Roerig, D. L. & Pederson, T. C. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1133–1137

Beauchamp, C. & Fridovich, I. (1970) *J. Biol. Chem.* **245**, 4641–4646

Brown, S. B. & King, R. F. G. J. (1976) *Biochem. Soc. Trans.* **4**, 197–201

Coburn, R. F., Danielson, G. K., Blakemore, W. S. & Forster, R. E., II (1964) *J. Appl. Physiol.* **19**, 510–515

De Matteis, F. (1971) *Biochem. J.* **124**, 767–777

De Matteis, F. & Sparks, R. G. (1973) *FEBS Lett.* **29**, 141–144

De Matteis, F. & Unseld, A. (1976) *Biochem. Soc. Trans.* **4**, 205–209

Dresel, E. I. B. & Falk, J. E. (1954) *Biochem. J.* **56**, 156–163

Estabrook, R. W., Franklin, M., Baron, J., Shigematsu, A. & Hildebrandt, A. (1971) in *Drugs and Cell Regulation* (Mihich, E., ed.), pp. 227–254, Academic Press, New York and London

Guzelian, P. S. & Bissell, D. M. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1393

Kikuchi, G. & Yoshida, T. (1976) *Ann. Clin. Res.* **8**, *Suppl.* **17**, 10–17

Labbe, R. F. & Nishida, G. (1957) *Biochim. Biophys. Acta* **26**, 437

Lamb, A. B., Scalione, C. C. & Edgar, G. (1922) *J. Am. Chem. Soc.* **44**, 738–757

Landaw, S. A. & Winchell, H. S. (1966) *J. Nucl. Med.* **7**, 696–707

Landaw, S. A., Callahan, E. W., Jr. & Schmid, R. (1970) *J. Clin. Invest.* **49**, 914–925

Levin, W., Lu, A. Y. H., Jacobson, M., Kuntzman, R., Poyer, J. L. & McCay, P. B. (1973) *Arch. Biochem. Biophys.* **158**, 842–852

Maines, M. D. & Kappas, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4293–4297

Masters, B. S. S. & Schacter, B. A. (1976) *Ann. Clin. Res.* **8**, *Suppl.* **17**, 18–27

McCord, J. M. & Fridovich, I. (1968) *J. Biol. Chem.* **243**, 5753–5760

McDonagh, A. F., Pospisil, R. & Meyer, U. A. (1976) *Biochem. Soc. Trans.* **4**, 297–298

Nishibayashi, H., Omura, T., Sato, R. & Estabrook, R. W. (1968) in *Structure and Function of Cytochromes* (Okunuki, K., Kamen, M. D. & Sekuzu, I., eds.), pp. 658–665, University Park Press, Baltimore and Manchester

O'Carra, P. (1975) in *Porphyrins and Metalloporphyrins* (Smith, K. M., ed.), pp. 123–153, Elsevier Scientific Publishing Co., Amsterdam

Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378

Paul, K.-G., Theorell, H. & Åkeson, Å. (1953) *Acta Chem. Scand.* **7**, 1284–1287

Poyer, J. L. & McCay, P. B. (1971) *J. Biol. Chem.* **246**, 263–269

Riely, C. A., Cohen, G. & Lieberman, M. (1974) *Science* **183**, 208–210

Schacter, B. A., Marver, H. S. & Meyer, U. A. (1973) *Drug Met. Dispos.* **1**, 286–290

Schwartz, S. & Ikeda, K. (1955) in *Ciba Found. Symp.: Porphyrin Biosynthesis and Metabolism* (Wolstenholme, G. E. W., ed.), pp. 209–226, J. and A. Churchill, London

Sjöstrand, T. (1970) *Ann. N.Y. Acad. Sci.* **174**, 5–10

Tenhunen, R., Marver, H. S. & Schmid, R. (1968) *Proc. Nat. Acad. Sci. U.S.A.* **61**, 748–755

Unseld, A. & De Matteis, F. (1976) in *Porphyrins in Human Diseases* (Doss, M., ed.), pp. 71–75, S. Karger A.-G., Basel

White, P. (1970) *Ann. N.Y. Acad. Sci.* **174**, 23–31

Wilbur, K. M., Berneim, F. & Shapiro, O. W. (1949) *Arch. Biochem.* **24**, 305–313

Wills, E. D. (1969) *Biochem. J.* **113**, 325–332

Wolff, D. G. & Bidlack, W. R. (1976) *Biochem. Biophys. Res. Commun.* **73**, 850–867