Ring Hydroxylation of Di-*t*-butylhydroxytoluene by Rat Liver Microsomal Preparations

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3,5-Di-*t*-butylhydroxytoluene (compound I) was converted into 4-hydroperoxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dienone (compound II), 4-hydroxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dienone (compound III) and 2,6-di-*t*-butyl-4-hydroxymethylphenol (compound IV) by rat liver microsomal preparations in the presence of NADPH and air. The oxidation of compound (I) by *m*-chloroperbenzoic acid also produced the same compounds. These results suggest that hydroperoxide can be an intermediate in aromatic hydroxylation and that biological oxygenations resemble per-acid reactions.

Large amounts of 3,5-di-*t*-butyl-4-hydroxytoluene (BHT; compound I) are used in many parts of the world as an antioxidant in processed food products, cosmetics and pharmaceuticals. Despite numerous biological and pathological studies which indicate the harmless nature of this compound (Hathway, 1966; Frawley *et al.*, 1965), its safety as an ingestible compound remains questionable (Milner, 1967; Brown *et al.*, 1959). Two groups of metabolic products of compound (I) have been identified. The first includes 3,5,3',5'-tetra-*t*-butyl-4,4'-dihydroxyl-1,2-diphenylethane (BHT-dimer; compound V) and its corresponding quinone (3,5,3',5'-tetra-*t*-butylstilbene-4,4'-quinone; BHT-dimer-quinone; compound VI) (Akagi & Aoki, 1962; Lodomery *et al.*, 1967); the second consists of the benzyl alcohol of compound (I), i.e. 2,6-di-*t*-butyl-4-hydroxymethylphenol (compound IV) (Aoki, 1962; Wright *et al.*, 1965), its aldehyde (Daniel *et al.*, 1967), and the corresponding benzoic acid (Dacre, 1961; Gilbert & Golberg, 1967). Also, the oxidation of a *t*-butyl substituent of compound (I) in rabbits has been reported (Daniel *et al.*, 1968) but not confirmed (Holder *et al.*, 1970).

In addition to the above mentioned compounds, it seemed reasonable to postulate that ring-oxygenated metabolites would be formed. Such ring metabolites

could potentially play an important biological role and serve to clarify the mechanism of oxygenation. In the present paper we describe the isolation of a ring-hydroxylated metabolite of compound (I), 4-hydroxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dienone (compound III) and its presumed immediate precursor, 4-hydroperoxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dienone (compound II) after incubation *in vitro* with rat liver microsomal oxygenase(s) (Scheme 1).

Materials and Methods

Isolation of rat liver microsomal preparations

Male Charles River rats weighing 120–180 g were used in these experiments. Liver microsomal preparations were made by the usual method of differential centrifugation. The liver of the decapitated animal was perfused *in situ* with 50 ml of 0.15 M-KCl solution. After removal from the carcass, the liver was cut into pieces and homogenized in 3 vol. (v/w) of 0.15 M-KCl. The homogenate was centrifuged for 15 min at 600 g in a Sorvall Superspeed RC2-B refrigerated centrifuge. The supernatant, after removal of the white waxy layer, was centrifuged for

Scheme 1. Proposed oxygenative pathway of compound (I)

20 min at 10800g. The 10800g supernatant was recentrifuged for 60 min at 105000g in a Beckman-Spinco model L preparative ultracentrifuge. The pellets obtained were rinsed once with 0.1M-sodium phosphate buffer, pH 7.4, suspended in buffer and centrifuged at 105000g for 60 min. The pellets were rinsed again with buffer, suspended in the phosphate buffer and stored at 0–5°C and used within 3 days. All the preparative procedures were carried out at 0–5°C. Microsomal protein content was measured by the biuret method (Robinson & Hogden, 1940) with crystalline bovine serum albumin as the standard. The cytochrome *P*-450 and *P*-420 contents of the microsomal preparations were determined (Omura & Sato, 1964a,b).

Qualitative and quantitative determinations

Analysis of the reaction mixture was done by using a Varian Aerograph gas-liquid chromatograph equipped with a flame-ionization detector. A glass column (150 cm × 6.5 mm outer diam.) was packed with 3% SE-30 methyl silicone on 100/120 mesh Var-A-Port 30 powder. N₂ was used as the carrier gas, at a flow rate of 25–30 ml/min. The temperature was kept at 110°C until the emergence of compound (II), then was programmed for a 20°C/min increase to 170°C, at which temperature compound (IV) emerged. A column of the same dimension packed with 2% XE-60 silicone nitrile on 80–100 mesh Gas-Chrom Q has also been used with similar results at temperatures of 100°C and 150°C. T.l.c. was carried out on alumina plates with chloroform as the solvent system. The compounds were located under u.v. light at 253.7 nm. This procedure was adopted primarily to detect the presence of dimers that could not be separated by g.l.c. (Ingold, 1963).

Incubation mixtures and extraction procedures

Reaction mixtures containing liver microsomal preparations, NADP⁺, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, compound (I) in acetone, and 0.1M-sodium phosphate buffer, pH 7.4, were incubated at 18°C with constant shaking at 110–120 oscillations/min (Fouts, 1970) in a Dubnoff metabolic shaking incubator. After incubation, 0.2 ml of conc. HCl was added before immediate extraction with peroxide-free diethyl ether (Gilbert & Golberg, 1967). Methyl palmitate was added to serve as an internal standard. The ether extract (60 ml) was dried over anhydrous Na₂SO₄ and evaporated to about 3 ml under N₂. A portion of the ether extract was subjected to t.l.c. primarily to detect the dimer and its quinone. The rest of the extract or, in most cases, the total extract was transferred into a small test-tube and evaporated to dryness under N₂. The

residue was dissolved in chloroform and promptly subjected to g.l.c. analysis.

Difference spectrum of compound (I)

Difference spectra were determined by recording the change in absorbance as a function of wavelength between microsomal preparations with compound (I) and microsomal preparations without the substrate in a Cary 14 recording spectrophotometer (Remmer *et al.*, 1966; Imai & Sato, 1966; Schenkman *et al.*, 1967).

Per-acid oxidation

Compound (I) (76 μmol) and *m*-chloroperbenzoic acid (300 μmol) in 30 ml of methylene chloride were first flushed with N₂ and then refluxed for various lengths of time. Portions were washed with 1M-Na₂CO₃ to remove acidic components. A portion of each sample was subjected to g.l.c. for analysis.

Chemicals

Glucose 6-phosphate, NADP⁺, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride) was a gift from Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A. Compound (I) was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Compound (II) (m.p. 115–116°C) and compound (III) (m.p. 112–113°C) were synthesized by the method of Kharash & Joshi (1957). Compound (IV) (m.p. 138–139°C) was prepared by the method of Nystrom & Brown (1947). All of these compounds were kept at –10° to –15°C before use. On t.l.c. and g.l.c. the compounds (I), (II), (III) and (IV) all appeared to be pure and showed up as single components.

Results

Compound (I) showed a difference spectrum with the microsomal enzyme preparations with a peak at 384 nm, trough at 419.5 nm and isosbestic point at 404 nm. A typical type-I curve (Schenkman *et al.*, 1967), indicating an enzyme-substrate interaction, was obtained.

All the compounds were quantitatively determined by g.l.c. with the SE-30 column system, as they could be separated without decomposition below 120°C. Compound (IV) was stable and required a higher temperature for emergence within a reasonable length of time (Fig. 1). Although the XE-60 column was used as a routine to confirm the results it was not used for quantitative measurements, because there was some decomposition of compound (II) on this

Fig. 1. Retention times of compounds (I), (II), (III) and (IV) and palmitate methyl ester on a column of SE-30

For details see the Materials and Methods section.

Fig. 2. Retention times of compounds (I), (II), (III) and (IV) and palmitate methyl ester on column XE-60

For details see the Materials and Methods section.

column even at a slightly lower temperature (Fig. 2). Its use in conjunction with the SE-30 column was advantageous because the relative retention times for some compounds were reversed and could be used as a further means of identification. The presence of compound (V) and compound (VI) was confirmed by t.l.c. and spectrophotometric determination. Although no attempt was made at quantitative measurement, it was apparent that more compound (V) and compound (VI) were obtained with longer incubation times or with larger amounts of microsomal enzymes. Compound (V) was easily converted into compound (VI) when not protected from light and air. Indeed, compound (II), compound (III) and compound (IV) in solution were slowly converted into compound (VI) on being left on the shelf.

In these experiments we have found, besides compound (IV), compound (V) and compound (VI) as reported by others (Hathway, 1966) and also compound (II) and compound (III) (Fig. 3). Even at 18°C and with relatively little microsomal protein, maximum product formation occurred within 5–10 min. The pH optimum was between 7.2 and 7.8, with a gradual decrease in activity on both sides of this pH range. As this was a multiple-product reaction, the pH profile was of limited value. O₂ was

Fig. 3. Effect of incubation time on the formation of compounds (II), (III) and (IV) from compound (I) by rat liver microsomal proteins

The incubation mixtures (5.0 ml) containing compound (I) (2.3 μ mol), α -NADPH-generating system composed of glucose 6-phosphate (26.9 μ mol), NADP⁺ (2.7 μ mol), and glucose 6-phosphate dehydrogenase (5 units), microsomal proteins (18 mg), and sodium phosphate buffer, pH 7.4, (500 μ mol) were incubated at 18°C for time indicated. Δ , Compound (II); $\blacksquare$, compound (III); $\bullet$, compound (IV).

required and CO inhibited the reactions (Table 1). The residual activity with helium was probably due to dissolved O₂ or enzyme-bound O₂ that was not or could not be removed by flushing the incubation mixture. The study of the subcellular localization of this ring-oxygenation activity revealed that it was in the microsomal fraction and thus was probably due to the microsomal oxygenase. The results also showed that there was reactivity, particularly hydroperoxidation, with boiled microsomal preparations. These activities were presumably catalysed by metal ions or complexes. The magnitude of such activities as compared with enzyme oxygenation varied from experiment to experiment. In our treatment of the results, such activities were taken into consideration whenever applicable. It is our experience that readily oxygenated substrates, (e.g. compound I), tend to be more extensively attacked by non-enzymatic catalysts.

The formation of compound (II), compound (III) and compound (IV) required NADPH (Fig. 4). Non-enzymic oxygenation was negligible, except for compound (II). The reaction rate increased with increased protein or substrate concentration, as could be expected (Figs. 5 and 6). As these products were readily further metabolized, kinetic studies were of little value. This was clearly indicated in Fig. 3. SKF 525-A, a typical liver microsomal oxygenase

Table 1. Requirement of O_2 and effect of CO on compound (I) ring-hydroxylation in rat liver microsomal preparations

The reaction mixtures (5.0ml) containing compound (I) ($2.3\mu\text{mol}$), glucose 6-phosphate ($26.0\mu\text{mol}$), NADP^+ ($2.7\mu\text{mol}$), glucose 6-phosphate dehydrogenase (5 units, where one unit will oxidize $1.0\mu\text{mol}$ of glucose 6-phosphate to 6-phosphogluconate/min at pH 7.4 at 25°C in the presence of NADP^+), liver microsomal protein ($7.5\text{--}10\text{mg}$) and sodium phosphate buffer, pH 7.4 ($500\mu\text{mol}$), were bubbled with gases as indicated and were incubated at 18°C for 10 min. The reaction mixture, without microsomal preparations, was placed in the body of Thunberg tubes and the microsomal preparations were placed in the side-arms.

Gas	Metabolite produced (%)			
	Compound (II)	Compound (III)	Compound (IV)	Total oxygenated product
Air	100	100	100	100
Helium	8.5	3.2	5.2	5.6
$\text{CO} + \text{O}_2$ (80:20)	94.3	46.0	69.5	77.5

Fig. 4. Effect of NADPH on the transformation of compound (I) by rat liver microsomal preparations

The reaction mixtures (5.0ml) containing compound (I) ($0.92\mu\text{mol}$), liver microsomal proteins (6mg), $\alpha\text{-NADPH}$ -generating system composed of glucose 6-phosphate ($26.9\mu\text{mol}$), NADP^+ ($5.38\mu\text{mol}$), and glucose 6-phosphate dehydrogenase (5 units), and sodium phosphate buffer, pH 7.4, ($500\mu\text{mol}$) were incubated at 18°C for 10 min. $\square$, Complete system; $\blacksquare$, system with NADPH omitted.

inhibitor (Brodie, 1956; Brodie *et al.*, 1958) inhibits compound (II), compound (III) and compound (IV) formation (Table 2) (Gilbert & Golberg, 1967). More meaningful information about the sequence of events could not be secured at the present state of our understanding of these reactions.

Three unidentified peaks appeared on the gas-liquid chromatogram. Two were close to compound (I) and one to compound (IV) on the SE-30 column.

Fig. 5. Effect of microsomal protein concentration on the transformation of compound (I) by rat liver microsomal preparations

The reaction mixtures (5.0ml) containing compound (I) ($2.3\mu\text{mol}$), $\alpha\text{-NADPH}$ -generating system composed of glucose 6-phosphate ($26.9\mu\text{mol}$), NADP^+ ($1.35\mu\text{mol}$), and glucose 6-phosphate dehydrogenase (5 units), microsomal proteins (as indicated), and sodium phosphate buffer, pH 7.4, ($500\mu\text{mol}$) were incubated at 18°C for 10 min. $\bullet$, Compound (II); $\blacksquare$, compound (III); $\blacktriangle$, compound (IV).

They appeared to be oxygenated products, as both SKF 525-A and CO inhibited, and O_2 and NADPH were required for their formation. Quantitatively, as judged by the peak area, they were comparable with known oxygenation products. Their identity and importance in metabolism remain to be clarified.

On treatment with *m*-chloroperbenzoic acid, compound (I) yielded compound (II), compound (III) and compound (IV) and some unknown compounds as shown by g.l.c. The products increased

Fig. 6. Effect of substrate concentration on the transformation of compound (I) in rat liver microsomal preparations

The reaction mixtures (5.0ml) containing compound (I) (as indicated), α -NADPH-generating system as in Fig. 3, microsomal proteins (14mg), and sodium phosphate buffer, pH 7.4 (500 μ mol), were incubated at 18°C for 10 min. ●, Compound (II); ■, compound (III); ▲, compound (IV).

Table 2. Effect of SKF 525-A on compound (I) metabolism

The reaction mixtures (5.0ml) containing compound (I) (6.37 μ mol), glucose 6-phosphate (26.9 μ mol), NADP⁺ (2.7 μ mol), glucose 6-phosphate dehydrogenase (5 units), liver microsomal proteins (14mg), SKF 525-A (0.98 μ mol), and sodium phosphate buffer, pH 7.4, (480 μ mol) were placed in a 25 ml flask and incubated at 18°C for 10 min. —, Undetectable.

Products	Metabolite produced (nmol/mg of protein)	
	Control	+SKF 525-A
Compound (II)	13.9	—
Compound (III)	22.1	16.5
Compound (IV)	26.3	12.0

with time of reflux. Of the compound (I) used 7.7% was converted into compound (II) after 14h as shown in Table 3. No products could be found when the per-acid was omitted. When the reaction was done on a preparative scale, crystalline compound (II), that was identical with the authentic compound according to its m.p., mixed m.p., u.v. spectrum, R_f on t.l.c., and retention time on g.l.c., could be obtained.

Discussion

Our working hypothesis for biological oxygenation by cytochrome P-450 oxygenases is that an initial attack of 1 mol of O₂ occurs, followed by reduction

Table 3. Non-enzymic conversion of compound (I) into compound (II), compound (III) and compound (IV) by *m*-chloroperbenzoic acid

The reaction mixture contained 76 μ mol of compound (I) and 300 μ mol of *m*-chloroperbenzoic acid in 30ml of methylene chloride (see the Materials and Methods section).

Reflux time (h) ... Products	Conversion of compound (I) into product (%)				
	0	1	3	9	14
Compound (II)	0	3.6	4.5	5.3	7.7
Compound (III)	0	4.7	7.2	9.0	11.4
Compound (IV)	0	2.4	3.0	5.6	6.8

or by other reactions that cause oxygen-oxygen bond fission. This concept is in part based on previous work (Chen & Lin, 1968, 1969; Lin & Chen, 1969) and on known non-enzymic radical oxygenation reactions. The reacting species may be an activated ground-state O₂ molecule, a biradical, or superoxide (Strobel & Coon, 1971), both of which may be written as Enz-OO[•]. Judging from the product formed by oxygenases, singlet oxygen may be excluded, as unsaturated compounds yield epoxide (Bloom & Shull, 1955). There is sufficient evidence to indicate that the 'activated' oxygen species can react in the same manner as per-acids. Besides the epoxide formation just mentioned, an enzyme isolated from the fungus *Cylindrocarpum radiculicola* is capable of inserting an oxygen atom into progesterone in the formation of testosterone acetate (Rahim & Sih, 1966). Both are known per-acid-type reactions. The so-called non-specific oxygenases of liver microsomal preparations may be very specific in that the positions of a substrate most vulnerable to attack by radical reactions usually acquire O₂. Chemically, the *para* position of phenols is prone to radical attacks. Biologically, it was found that phenol was converted into *p*-quinol *in vivo* (Porteous & Williams, 1949). Westerfeld (1940) suggested that 10-hydroxy-3,17-dioxo-oestra-1,4-diene could be a metabolite of oestrone. Hecker & Mueller (1958) were able to show that 5,6,7,8-tetrahydro-2-[8-¹⁴C]naphthol was oxygenated to tetralin-*p*-quinol by rat liver microsomal preparations, NADPH and air. Further, oestradiol-17 β yielded 17 β -hydroxyoestra-*p*-10 β -quinol (Hecker & Zayed, 1961). Should hydroperoxide be the initial oxygenation product also of phenols, one may predict that compound (I) should yield compound (II) and compound (III) along with compound (IV), as shown in the present paper. Compound (IV) had previously been isolated and identified (Kharash & Joshi, 1957; Hathway, 1966). It is to be expected, as

the toluene methyl group is known to be highly reactive toward radical and ionic attacks. The formation of compound (II) by liver microsomal oxygenase further confirmed our contention that hydroperoxides are the initially detectable intermediates of hydroxylation. This then suggests that compound (I) might not be quite as harmless as it has been purported to be. Hydroperoxides are usually highly reactive and may react with cellular constituents before the hydroperoxides are further metabolized. Indeed, many of the possible radical intermediates may well be equally or even more reactive and potentially toxic according to the following reactions:

Under these circumstances, biotransformation may be associated with bioactivation. Preliminary studies in our laboratories with ^3H -labelled compound (I) showed that it was bound covalently to rat liver microsomal protein in the presence of NADPH and O_2 .

Compound (I) is an antioxidant in the chemical sense. Besides processed food, pharmaceuticals and cosmetics, it is also used in petroleum products and polymers. It is an equally efficient antioxidant biologically. Harman (1968) concluded that its anti-oxidation properties, like α -tocopherol, prevented aging of rats. It thus follows that biological and chemical oxygenation and autoxidation could be mechanistically the same. Boiled enzyme preparations, which still contain metal ions and possibly metal chelates, yielded small amounts of oxygenation products. That oxygenation reactions of liver homogenates were confined in the microsomal fraction, required NADPH and O_2 and were inhibited by CO and SKF 525-A seems to support the fact that enzymic reactions were involved.

Fig. 3 indicates the high reaction rate observed. The maximum is reached within 5–10 min at 18°C instead of the usual 30 min or longer at 37°C for other substrates. This parallels the high rate of reaction of compound (I) with O_2 in alkaline ethanolic solution as described by Kharash & Joshi (1957). More interesting is the observation that compound (III) appears to have a shorter life than compound (IV). Both appear to be converted into other compounds at higher rates than is compound (II). One can explain that compound (IV) was further oxidized to its benzaldehyde and benzoic acid by an oxygenase rather than by a dehydrogenase, as NADPH and O_2 were present in the reaction mixture. This is plausible as Lieber & DeCarli (1968) have shown

that ethanol was oxidized to acetaldehyde by a rat liver microsomal oxygenase. The disappearance of compound (III) was more puzzling. It could be dehydrated back to compound (I) or further oxygenated at a different position such as the toluene methyl group to yield a *vic*-diol. The results did not permit us to delineate the sequence of events, neither were we able to suggest the final metabolic products, with the possible exception of the benzoic acid analogue. Compound (VI) could be another terminal product. It might be resistant to further oxygenation.

As oxygenases caused the formation of lactones (Prairie & Talalay, 1963) and esters (Rahim & Sih, 1966) from ketones, and epoxides from unsaturated compounds (Bloom & Shull, 1955), it seems logical to suggest that the oxygenase-activated O_2 behaves as a per-acid. Should compound (II) be the initial product of oxygenase on compound (I), one would then also expect that a per-acid would give compound (II). *p*-Hydroxytoluene and its derivatives give quinols (Kumagai & Wolfenstein, 1908; Goodwin & Witkop, 1957), which could be products of unstable hydroperoxides. The reaction of compound (I), which yields a stable hydroperoxide, with *m*-chloroperbenzoic acid would provide a meaningful mechanistic model for oxygenases. Our experiments, which were performed at a few degrees above the physiological temperature, yielded 7.7% of compound (II). If one considers compound (III) to be a product of compound (I), then the yield was as high as 19%, and all the starting compound treated similarly was recovered unchanged when the per-acid was omitted. Further, as with enzymic reactions, compound (IV) was isolated. These results support the hypothesis that in oxygenase reactions the activated oxygen reacts as $-\text{OO}-$ as opposed to $-\text{O}-$, and this enzyme-oxygen complex reacts as a per-acid.

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References

- Akagi, M. & Aoki, I. (1962) *Chem. Pharm. Bull.* **10**, 101–105
- Aoki, I. (1962) *Chem. Pharm. Bull.* **10**, 105–112
- Bloom, B. M. & Shull, G. D. (1955) *J. Amer. Chem. Soc.* **77**, 5767–5768
- Brodie, B. B. (1956) *J. Pharm. Pharmacol.* **8**, 1–17
- Brodie, B. B., Gillette, J. R. & LaDu, B. M. (1958) *Annu. Rev. Biochem.* **27**, 427–454
- Brown, W. D., Johnson, A. R. & O'Halloran, M. W. (1959) *Aust. J. Exp. Biol. Med. Sci.* **37**, 533–548
- Chen, C. & Lin, C. C. (1968) *Biochim. Biophys. Acta* **170**, 366–374
- Chen, C. & Lin, C. C. (1969) *Biochim. Biophys. Acta* **184**, 634–640

- Dacre, J. C. (1961) *Biochem. J.* **75**, 758–766
- Daniel, J. W., Gage, J. C., Jones, D. I. & Stevens, M. A. (1967) *Food Cosmet. Toxicol.* **5**, 475–479
- Daniel, J. W., Gage, J. C. & Jones, D. I. (1968) *Biochem. J.* **106**, 783–790
- Fouts, J. R. (1970) *Toxicol. Appl. Pharmacol.* **16**, 48–65
- Frawley, J. P., Kohn, F. E., Kay, J. W. & Calandra, J. C. (1965) *Food Cosmet. Toxicol.* **3**, 377–386
- Gilbert, D. & Golberg, L. (1967) *Food Cosmet. Toxicol.* **5**, 481–490
- Goodwin, S. & Witkop, B. (1957) *J. Amer. Chem. Soc.* **79**, 179–185
- Harman, D. (1968) *J. Gerontol.* **23**, 476–482
- Hathway, D. E. (1966) *Advan. Food Res.* **15**, 1–56
- Hecker, E. & Mueller, G. C. (1958) *J. Biol. Chem.* **233**, 991–996
- Hecker, E. & Zayed, S. M. A. D. (1961) *Hoppe-Seyler's Z. Physiol. Chem.* **325**, 209–223
- Holder, G. M., Ryan, A. J., Watson, T. R. & Wiebe, L. I. (1970) *J. Pharm. Pharmacol.* **22**, 375–376
- Imai, Y. & Sato, R. (1966) *Biochem. Biophys. Res. Commun.* **22**, 620–626
- Ingold, K. U. (1963) *Can. J. Chem.* **41**, 2807–2815
- Kharash, M. S. & Joshi, B. S. (1957) *J. Org. Chem.* **22**, 1439–1443
- Kumagai, T. & Wolffenstein, R. (1908) *Ber. Deut. Chem. Ges.* **41**, 297–300
- Lieber, C. S. & DeCarli, L. M. (1968) *Science* **162**, 917–918
- Lin, C. C. & Chen, C. (1969) *Biochim. Biophys. Acta* **192**, 133–135
- Lodomery, L. G., Ryan, A. J. & Wright, S. E. (1967) *J. Pharm. Pharmacol.* **19**, 383–387
- Milner, S. M. (1967) *Nature (London)* **216**, 557–560
- Nystrom, R. F. & Brown, W. G. (1947) *J. Amer. Chem. Soc.* **69**, 1197–1199
- Omura, T. & Sato, R. (1964a) *J. Biol. Chem.* **239**, 2370–2378
- Omura, T. & Sato, R. (1964b) *J. Biol. Chem.* **239**, 2379–2385
- Porteous, J. W. & Williams, R. T. (1949) *Biochem. J.* **44**, 46–55
- Prairie, R. L. & Talalay, P. (1963) *Biochemistry* **2**, 203–208
- Rahim, M. A. & Sih, C. J. (1966) *J. Biol. Chem.* **241**, 3615–3623
- Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J., Narasimhula, S., Cooper, D. Y. & Rosenthal, O. (1966) *Mol. Pharmacol.* **2**, 187–190
- Robinson, H. W. & Hogden, C. G. (1940) *J. Biol. Chem.* **135**, 707–725
- Schenkman, J. B., Remmer, H. & Estabrook, R. W. (1967) *Mol. Pharmacol.* **3**, 113–123
- Strobel, H. H. & Coon, M. J. (1971) *J. Biol. Chem.* **246**, 7826–7829
- Westerfeld, W. W. (1940) *Biochem. J.* **34**, 51–58
- Wright, A. S., Akintonwa, D. A. A., Crowne, R. S. & Hathway, D. E. (1965) *Biochem. J.* **97**, 303–310