The Distribution of the Components of Mixed-Function Oxidase between the Rough and the Smooth Endoplasmic Reticulum of Liver Cells

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Mixed-function oxidase activity, when measured by the *N*-demethylation of ethylmorphine or the hydroxylation of aniline, is significantly higher in the smooth hepatic endoplasmic reticulum than in the rough. In the rabbit the smooth membrane/rough membrane activity ratios are significantly greater than 1 whether the activities are expressed per g. of liver (ratio 5), per mg. of protein (ratio 3–5), per μ g. of phospholipid phosphorus (ratio 2), per unit of cytochrome *P*-450 (ratio 1.7) or per unit of NADPH–cytochrome *c* reductase activity (ratio 2). On the other hand, if the activities are normalized to the NADPH–cytochrome *P*-450 reductase, there is no significant difference between the rough and smooth membranes. These results suggest that, in the rabbit, the rate-limiting step is the reduction of cytochrome *P*-450. In contrast, in the rat the difference in activities can be explained by differences in the concentration of cytochrome *P*-450.

Cytochrome *P*-450 is considered to be an important component of the mixed-function oxidases involved in the hydroxylation of steroids and many foreign compounds (Estabrook, Cooper & Rosenthal, 1963; Cooper, Levine, Narasimhulu, Rosenthal & Estabrook, 1965). In the presently accepted scheme (Gillette, 1966) NADPH cyclically reduces the cytochrome *P*-450 through the enzyme NADPH–cytochrome *c* reductase. The reduced cytochrome *P*-450 then catalyses the hydroxylation

of the substrate and in the process returns to the oxidized state (Scheme 1).

Several workers have reported that there is greater mixed-function oxidase activity in the smooth endoplasmic reticulum than in the rough, whether the activity is expressed per g. wet wt. of liver or per mg. of protein (Fouts, 1961; Remmer & Merker, 1965; Mason, Yamano, North, Hashimoto & Sakagishi, 1965; Gram, Rogers & Fouts, 1967). Further, we have found in the work reported below

Scheme 1. Sequence of reactions involved in the mixed-function oxidases of hepatic endoplasmic reticulum.

that, in the rabbit liver preparation, this difference, though less, is still marked when the enzymic activities are expressed either per unit of phospholipid, which is presumably a good index of the amount of membrane in the preparation, or per unit of cytochrome *P*-450. Both these methods of normalizing the activity eliminate the apparent decrease in activity of the rough membrane, which would be due to the dilution of the enzymically active protein of the membrane with the inactive protein of the ribosomes. This lower activity per unit of membrane and per unit of cytochrome *P*-450 suggests that in rough endoplasmic reticulum cytochrome *P*-450 is not as readily reduced as that found in the smooth; and indeed our results are compatible with this hypothesis.

METHODS

Fed untreated male New Zealand rabbits (2.0–2.5 kg.) and Sprague–Dawley rats (160 g.) from the National Institutes of Health colony were killed by a blow to the head, and the livers were excised and chilled on ice.

Fractions of hepatic smooth and rough microsomes were prepared by the method of Dallner (1963). The livers were washed in chilled 0.25 M-sucrose, and 15 g. of liver was homogenized in 0.25 M-sucrose (15 ml.) in a motor-driven Teflon–glass homogenizer with 0.10–0.15 mm. clearance. The volume of the homogenate was brought to 60 ml. with 0.25 M-sucrose and the mixture was centrifuged at 10000 *g* for 20 min. in a Sorvall RC-2 centrifuge (SS-34 rotor). A portion of the supernatant (9–85 ml.) was mixed with 1 M-CsCl (0.15 ml.), and 7.5 ml. of this mixture was then layered over 1.30 M-sucrose–0.015 M-CsCl (4–5 ml.). The gradients were centrifuged at 105000 *g*_{av} for 143 min. in a Spinco model L centrifuge (no. 40 rotor). The layer around the interface was aspirated, the volume brought to 9 ml. with 0.25 M-sucrose and the sample centrifuged at 105000 *g*_{av} for 60 min. The pellet of smooth microsomes formed in each tube was suspended in 0.15 M-KCl–0.02 M-tris (3.75 ml.). The remaining 1.30 M-sucrose was aspirated from the gradient tubes, and the pellets of rough microsomes were resuspended in 0.15 M-KCl–0.02 M-tris (3.75 ml.). The purities of the fractions have been well established by previous electron-micrographic studies, which indicate 10–15% contamination of the pellet of rough microsomes with smooth microsomes (Gram *et al.* 1967; Dallner, 1963).

Protein was determined by the method of Sutherland, Cou, Haynes & Olsen (1949).

The concentrations of cytochrome *P*-450 were determined by gassing a sample containing 3 mg. of protein/ml. of 0.15 M-KCl–0.02 M-tris for 5 min. with CO that had been deoxygenated by passage through three gassing towers each containing 200 ml. of 0.05% sodium anthraquinone-2-sulphonate and 5% sodium dithionite in 0.1 N-NaOH. The absorption spectrum was recorded, in a Shimadzu MPS-50L spectrophotometer, against an ungassed sample before and after the addition of a small amount of solid dithionite to both samples. The extinction due to cytochrome *P*-450 was taken to be the difference $E_{450} - E_{490}$ before the addition of dithionite subtracted from the difference after the addition.

Binding of ethylmorphine, a type I ligand for microsomes (Remmer *et al.* 1966), was determined by the sequential addition of 4 μ l. portions of 0.15 M substrate to a sample of the microsomal preparation (3 ml.) containing 3 mg. of protein/ml. of 0.15 M-KCl–0.02 M-tris. The changes in the difference spectrum were recorded at 420 and 450 m μ in the Shimadzu MPS-50L spectrophotometer. The extinction due to the binding was determined as described above for cytochrome *P*-450. Nothing was added to the reference sample. Unfortunately, owing to the small changes in extinction (0.001–0.020) from 0.2 to 5 mm it was not possible to obtain a double inverse regression line with low variance. The binding data were therefore taken as the difference in extinction with 1 mM-ethylmorphine in the sample cuvette.

Phospholipids were extracted by the methanol–chloroform–water method of Bligh & Dyer (1959). The phospholipid phosphorus was determined by the method of Chen, Toribara & Warner (1956), in which dried samples were burned with a mixture of H₂SO₄ and HClO₄ and the phosphorus was determined with ascorbic acid–molybdc acid.

In the first experiment mixed-function oxidase activity was determined for both the hydroxylation of aniline and the *N*-demethylation of ethylmorphine; in later experiments only the latter reaction was studied. Duplicate incubations for both reactions were performed as follows. To a 20 ml. serum bottle was added 1 ml. of a solution containing tris–HCl buffer, pH 7.4 (150 μ moles), MgCl₂ (15 μ moles), glucose 6-phosphate (30 μ moles), NADP⁺ (1.5 μ moles) and glucose 6-phosphate dehydrogenase (2 units; 1 unit reduced 1 μ mole of NADP⁺/min. at pH 7.4 and 25°). This was followed by 1 ml. of either 3 mM-aniline hydrochloride or 3 mM-ethylmorphine in water and 1 ml. of the enzyme preparation containing 3 mg. of microsomal protein/ml. of 0.15 M-KCl–0.02 M-tris. These give a total volume of 3 ml. The samples were incubated at 37° for 10 min. (rats) or 20 min. (rabbits) in air. Under these conditions both reactions are linear with respect to time and protein concentration. The hydroxylation of aniline was stopped by placing the vessels in an ice bath. The amount of *p*-aminophenol formed was determined by ether extraction and the formation of phenyl-indophenol (Kato & Gillette, 1965). The *N*-demethylation of ethylmorphine was stopped by the addition of 5% (w/v) ZnSO₄ (1 ml.) to each vessel followed by a 3:1 (v/v) mixture of saturated solutions of Ba(OH)₂ and Na₂B₄O₇ (2 ml.). After centrifugation, formaldehyde was determined in the clear supernatant (3.0 ml.) by using 0.4 ml. of acetylacetone added to 4 M-ammonium acetate in 0.1 M-acetic acid (Nash, 1953).

The NADPH–cytochrome *c* reductase activity was determined by a modification of the method of Phillips & Langdon (1962). To a 10 mm. $\times$ 10 mm. spectrophotometric cuvette was added 1 ml. of the diluted microsomal preparation (300–500 μ g. of protein/ml. of tris), 1 ml. of 3 mM-KCN and 1 ml. of 0.15 M-oxidized horse heart cytochrome *c*. (The concentration of a 2.5 mg./ml. solution in water of oxidized horse heart cytochrome *c* was adjusted to 0.15 M by determining the change in E_{550} before and after the addition of dithionite to a 5:1 dilution of the cytochrome in water. The molar extinction coefficient was assumed to be 18700.) The cuvette was warmed for 5 min. to 37° in a Gilford 2000 recording spectrophotometer. With the slit set to 0.1 mm., the extinction at 550 m μ was offset to give a reading of zero on the chart. With the chart running, 50 μ l. of an NADPH-generating system, prepared by mixing

0.3M-tris-HCl buffer, pH 7.4 (0.2ml.), 1M-MgCl₂ (0.2ml.), sodium isocitrate (480 μ moles), NADP⁺ (30 μ moles) and isocitrate dehydrogenase (0.6ml. of 10mg./ml. of glycerol), was added. The reaction was determined from the initial velocity. This reaction was linear with respect to microsomal protein concentration and was saturated with respect to NADPH and oxidized cytochrome *c*.

The NADPH-cytochrome *P*-450 reductase activity was also determined as follows: 3ml. of a microsomal suspension (3mg. of protein/ml. of 0.15M-KCl-0.02M-tris) was added to an Aminco anaerobic spectrophotometric cuvette. Then CO, which had been deoxygenated as described above, was bubbled into the suspension for 5min. The plunger containing 50 μ l. of the same NADPH-generating system as that used in the cytochrome *c* reductase assay was then placed in the cuvette and the gas phase above the solution gassed for 3min. The cuvette was closed and warmed for 5min to 37° in a Gilford 2000 spectrophotometer. The extinction at 450m μ was offset so that the chart read zero and the plunger quickly depressed. The reaction rate was determined from the initial velocity. The reaction was linear with respect to microsomal protein and was saturated with respect to NADPH. In the last rabbit experiment, measurements of E_{450} and E_{490} were made both before the addition of NADPH and after the completion of the

reaction. The plunger was removed and some dithionite was added, and E_{450} and E_{490} were again measured. These data were used to calculate the NADPH-reducible cytochrome *P*-450 and total cytochrome *P*-450.

RESULTS

As shown in Table 1, the smooth microsomes from rabbits have significantly greater oxidase activity per g. wet wt. of liver than the rough. However, there is also more protein, cytochrome *P*-450 and phospholipid in the smooth microsomes, indicating that there are more smooth microsomes than rough in this preparation.

When these data are normalized for the concentration of microsomal protein, as in Table 1, there are still marked differences in the oxidase activity of the smooth and rough membranes. The smooth membrane/rough membrane activity ratios are similar to those observed by Gram *et al.* (1967).

If the data are normalized to the amount of phospholipid, which is an index of the amount of membrane present, there is still markedly more

Table 1. *Aniline hydroxylase activities, ethylmorphine N-demethylase activities and cytochrome P-450 concentrations in smooth and rough microsomes from the livers of adult male rabbits*

Preparations and assays are given in the text. Values are given as means $\pm$ S.E.M. of the results obtained with five animals.

	Aniline hydroxylase activity (nmoles of <i>p</i> -aminophenol formed/20 min.)			Ethylmorphine <i>N</i> -demethylase activity (nmoles of formaldehyde formed/20 min.)		
	Smooth microsomes (A)	Rough microsomes (B)	A/B ratio	Smooth microsomes (C)	Rough microsomes (D)	C/D ratio
Per g. wet wt. of liver	72.8 $\pm$ 7.2	15.1 $\pm$ 0.89	4.97 $\pm$ 0.71	244.7 $\pm$ 27.8	52.6 $\pm$ 4.2	4.68 $\pm$ 0.49
Per mg. of microsomal protein	5.09 $\pm$ 0.15	1.61 $\pm$ 0.10	3.12 $\pm$ 0.19	17.1 $\pm$ 0.9	5.60 $\pm$ 0.56	3.10 $\pm$ 0.21
Per μ g. of phospholipid P	0.781 $\pm$ 0.020	0.406 $\pm$ 0.025	1.94 $\pm$ 0.09	2.61 $\pm$ 0.10	1.43 $\pm$ 0.13	1.89 $\pm$ 0.15
Per $\Delta(E_{450} - E_{490})$	50.4 $\pm$ 3.1	30.5 $\pm$ 2.8	1.68 $\pm$ 0.09	168 $\pm$ 10.0	108 $\pm$ 15.3	1.63 $\pm$ 0.14

Table 2. *Activities in smooth and rough microsomes from the livers of adult male rabbits of ethylmorphine N-demethylase and NADPH-cytochrome c reductase and the change in extinction between 420 and 450m μ on the addition of 1 mM-ethylmorphine to a cuvette containing 3mg. of protein/ml. of potassium chloride-tris*

Preparations and assays are given in the text. Values are given as means $\pm$ S.E.M. of the results obtained with five animals.

	Smooth microsomes (A)	Rough microsomes (B)	A/B ratio
Ethylmorphine <i>N</i> -demethylase activity/mg. of protein (nmoles of formaldehyde formed/20 min./mg. of protein)	56.0 $\pm$ 2.25	11.2 $\pm$ 3.53	5.65 $\pm$ 0.80
$\Delta(E_{420} - E_{450})$	-0.011 $\pm$ 0.0018	-0.0087 $\pm$ 0.0012	1.39 $\pm$ 0.38
NADPH-cytochrome <i>c</i> reductase activity/mg. of protein (nmoles of cytochrome <i>c</i> reduced/20 min./mg. of protein)	0.085 $\pm$ 0.008	0.029 $\pm$ 0.007	3.53 $\pm$ 0.80
NADPH-cytochrome <i>c</i> reductase activity/unit of cytochrome <i>P</i> -450 [nmoles of cytochrome <i>c</i> reduced/min./ $\Delta(E_{450} - E_{490})$]	2006 $\pm$ 224	3414 $\pm$ 666	0.665 $\pm$ 0.105

oxidase activity associated with the smooth than with the rough membranes (Table 1). There is also a small, but significantly higher, concentration of cytochrome *P*-450 per unit 'membrane' in the smooth than in the rough membranes.

Even when the mixed-function oxidase activity is normalized to the concentration of cytochrome *P*-450 (Table 1), there is still significantly greater activity in the smooth than in the rough membranes. Although there is no question as to the importance of cytochrome *P*-450 in the mixed-function oxidases, these data clearly indicate that the concentration of this cytochrome is not the rate-limiting factor in these reactions.

Schenkman, Frey, Remmer & Estabrook (1967) have suggested that the rate of metabolism is proportional to the binding of the substrate to the microsomes. As shown in Table 2 this is not the case, since there is no significant difference between the preparations of rough and smooth microsomes in the spectral change on the addition of ethylmorphine. Within the limitations of this measurement, the relative binding capacities of the smooth and the rough microsomes seems to be related to the relative concentrations of cytochrome *P*-450 in each of the fractions. There seems little likelihood that the values for binding of ethylmorphine to the

smooth microsomes could have been 5.65 times (the factor necessary in this experiment to explain the difference in metabolism) that to the rough microsomes without it being apparent in these data.

Alternatively the NADPH-cytochrome *c* reductase activity could be sufficiently lower in the rough than in the smooth membranes to explain the differences in oxidase activity. Although the smooth membrane/rough membrane *N*-demethylase activity ratio in this experiment was 5.65 ± 0.80 , the ratio of NADPH-cytochrome *c* reductase activities was only 2.23 ± 0.22 (Table 2). Clearly the activity of NADPH-cytochrome *c* reductase is also not the rate-determining factor in oxidase activity. Further, the amount of this enzyme per unit of cytochrome *P*-450 is actually higher in the rough than in the smooth membranes (Table 2). This suggests that, if there is no intermediate between the NADPH-cytochrome *c* reductase and cytochrome *P*-450, the NADPH-cytochrome *P*-450 reductase activity should be greater in the rough than in the smooth membranes. Yet the reverse is the case, as there is markedly greater NADPH-cytochrome *P*-450 reductase activity in the smooth than in the rough membranes (Table 3). It has been

Table 3. *NADPH*-cytochrome *P*-450 reductase activity in smooth and rough microsomes from the livers of adult male rabbits

Preparation and assays are given in the text. Values are given as means $\pm$ s.e.m. of the results obtained with four (Expt. I) or six (Expt. II) animals.

	Cytochrome <i>P</i> -450 reductase activity ($\Delta E_{450}/\text{min.}/\text{mg. of protein}$)		
	Smooth microsomes (A)	Rough microsomes (B)	A/B ratio
Expt. I	0.697 ± 0.135	0.158 ± 0.039	4.67 ± 0.78
Expt. II	0.542 ± 0.101	0.114 ± 0.028	5.55 ± 1.01

Table 5. *Ethylmorphine N*-demethylase activity per unit of *NADPH*-cytochrome *P*-450 reductase activity in the smooth and rough microsomes from the livers of adult male rabbits

Preparation and assays are given in the text. Values are given as means $\pm$ s.e.m. of the results obtained with four (Expt. I) or six (Expt. II) animals.

	<i>Ethylmorphine N</i> -demethylase activity/unit of <i>NADPH</i> -cytochrome <i>P</i> -450 reductase activity (nmoles of formaldehyde formed/ $\Delta E_{450}/\text{min.}$)		
	Smooth microsomes (A)	Rough microsomes (B)	A/B ratio
Expt. I	4.67 ± 0.08	3.47 ± 0.47	1.37 ± 0.20
Expt. II	5.91 ± 0.72	6.51 ± 0.99	0.96 ± 0.14

Table 4. *Values of cytochrome P*-450 when reduced with *NADPH* and dithionite in a Gilford 2000 spectrophotometer for smooth and rough microsomes from the livers of adult male rabbits

Preparation and assays are given in the text. Values are given as means $\pm$ s.e.m. of the results obtained with six animals.

	Cytochrome <i>P</i> -450 [$\Delta(E_{450} - E_{490})/\text{mg. of protein}$]		
	Smooth microsomes (A)	Rough microsomes (B)	A/B ratio
Reduction with <i>NADPH</i>	0.107 ± 0.008	0.045 ± 0.007	2.54 ± 0.27
Reduction with dithionite	0.117 ± 0.006	0.057 ± 0.005	2.12 ± 0.17
Fraction of cytochrome <i>P</i> -450 reduced by <i>NADPH</i>	0.991 ± 0.061	0.771 ± 0.058	

Table 6. *Activity of ethylmorphine N-demethylase per mg. of protein and per unit of cytochrome P-450 for smooth and rough microsomes from the livers of adult male rats*

Preparation and assays are given in the text. Values are given as means $\pm$ S.E.M. of the results obtained with five animals.

	Smooth microsomes (A)	Rough microsomes (B)	A/B ratio
Ethylmorphine <i>N</i> -demethylase activity/mg. of protein (nmoles of formaldehyde formed/mg. of protein/10 min.)	62.9 $\pm$ 5.5	36.0 $\pm$ 3.9	1.78 $\pm$ 0.13
Ethylmorphine <i>N</i> -demethylase activity/unit of cytochrome P-450 [nmoles of formaldehyde formed/ $\Delta(E_{450} - E_{490})/10$ min.]	1228 $\pm$ 76	1126 $\pm$ 115	1.12 $\pm$ 0.10

observed (Gram, Gigon & Gillette, 1968) that the addition of ethylmorphine preparations increases the rate of cytochrome *P* 450 reduction. In preliminary experiments the addition of this substrate stimulated the reduction in both smooth and rough membranes without affecting the ratio of the rate in the smooth to that in the rough membranes.

Not only is the rate at which cytochrome *P*-450 is reduced lower in the rough membrane, but also the fraction of the total cytochrome *P*-450 that is reducible by NADPH is also significantly lower in the rough than in the smooth membrane (Table 4). In the smooth membrane almost all of the cytochrome *P*-450 is reducible by NADPH, whereas in the rough membrane only about 77% is so reducible.

Finally, if we now normalize the *N*-demethylase activity to the NADPH-cytochrome *P*-450 reductase activity, we observe values in the smooth membrane that do not differ significantly from those observed in the rough membrane (Table 5).

Unlike the rabbit, the difference between the activity found in the rough and the smooth microsomes from rat liver cells can be explained on the basis of different concentrations of cytochrome *P*-450 in each of the fractions (Table 6).

DISCUSSION

In the rabbit, there is a marked difference in mixed-function oxidase activity between the rough and the smooth endoplasmic reticulum membranes of liver cells. Our data suggest that this difference results from two factors. The first is that there is less of the cytochrome *P*-450 in the rough than in the smooth membrane. This is all that is required to explain the lower activity in the rough membrane of rat liver cells. Secondly, the cytochrome *P*-450 that is present in the rough membrane of rabbits is more slowly and less fully reduced than that present in the smooth membrane. Yet, when normalized to cytochrome *P*-450, the rough membrane actually

contains more NADPH-cytochrome *c* reductase, the enzyme presumed to catalyse the reduction of cytochrome *P*-450 by NADPH, than does the smooth membrane.

These results are similar to those observed in young animals by Dallner, Siekevitz & Palade (1965*a,b*). They found that, although the activity of NADPH-cytochrome *c* reductase and concentration of cytochrome *P*-450 in the hepatic microsomal fraction derived from rats in the first days of life were similar to those found in adult animals, the young animals had significantly lower oxidase activity. On this basis, they suggested that there is a carrier, 'X', between the NADPH-cytochrome *c* reductase and the cytochrome *P*-450.

It is not necessary to postulate a carrier. Another possibility is that the newly formed enzyme becomes activated on leaving the rough membrane so that it can now reduce, not only cytochrome *c*, but also its normal substrate, cytochrome *P*-450. This activation could occur (1) by the removal of some inhibiting factor, such as the ribosomes, (2) by the addition of some substituent, as in the transformation of phosphorylase *b* to phosphorylase *a*, or (3) by some structural alteration, such as the addition of phospholipid to the membrane.

Finally, the enzyme NADPH-cytochrome *c* reductase may not really be a component of the electron chain involved in the reduction of cytochrome *P*-450. This does not seem likely, although Omura, Sanders, Estabrook, Cooper & Rosenthal (1966) have shown that the enzyme from liver microsomes cannot catalyse the reduction of adrenal mitochondrial cytochrome *P*-450. This does not exclude the role of this enzyme in the reduction of hepatic microsomal cytochrome *P*-450, since the adrenal system differs in other ways from the system we have studied. In particular it contains a non-haem iron that is necessary for the reduction of the cytochrome *P*-450. Such a non-haem iron has not been demonstrated in the microsomal fraction from liver (Miyake, Mason & Langraf, 1967).

The data from the present work suggest that there is a defect in the rough endoplasmic reticulum similar to that found in young animals. This defect is found in the only portion of the membrane that is actively synthesizing and organizing the constituents that, on leaving the rough membrane, become the components of the far more active smooth membrane (Campbell, 1965). Clearly, this defect is not due to the absence of some cytoplasmic factor, since both membranes are bathed in exactly the same cytoplasm, nor to the failure to synthesize some necessary protein as may have been the case in the young animals. The problem of the exact nature of this defect still remains open.

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REFERENCES

- Bligh, E. G. & Dyer, W. J. (1959). *Canad. J. Biochem. Physiol.* **37**, 911.
- Campbell, P. N. (1965). *Progr. Biophys. molec. Biol.* **15**, 1.
- Chen, P. S., jun., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
- Cooper, D. Y., Levine, S., Narasimhulu, S., Rosenthal, O. & Estabrook, R. W. (1965). *Science*, **147**, 400.
- Dallner, G. (1963). *Acta path. microbiol. scand. Suppl.*, p. 166.
- Dallner, G., Siekevitz, P. & Palade, G. E. (1965a). *Biochem. biophys. Res. Commun.* **20**, 135.
- Dallner, G., Siekevitz, P. & Palade, G. E. (1965b). *Biochem. biophys. Res. Commun.* **20**, 142.
- Estabrook, R. W., Cooper, D. Y. & Rosenthal, O. (1963). *Biochem. Z.* **338**, 741.
- Fouts, J. R. (1961). *Biochem. biophys. Res. Commun.* **6**, 373.
- Gillette, J. R. (1966). *Advanc. Pharmacol.* **4**, 245.
- Gram, T. E., Gigon, P. L. & Gillette, J. R. (1968). *Biochem. biophys. Res. Commun.* **31**, 558.
- Gram, T. E., Rogers, L. A. & Fouts, J. R. (1967). *J. Pharmacol.* **155**, 479.
- Kato, R. & Gillette, J. R. (1965). *J. Pharmacol.* **150**, 279.
- Mason, H. S., Yamano, T., North, J. C., Hashimoto, Y. & Sakagishi, P. (1965). In *Oxidases and Related Redox Systems*, vol. 2, p. 879. Ed. by King, T. E., Mason, H. S. & Morrison, M. New York: John Wiley and Sons Inc.
- Miyake, Y., Mason, H. S. & Langraf, W. (1967). *J. biol. Chem.* **242**, 393.
- Nash, I. (1953). *Biochem. J.* **55**, 416.
- Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y. & Rosenthal, O. (1966). *Arch. Biochem. Biophys.* **117**, 660.
- Phillips, A. H. & Langdon, R. G. (1962). *J. biol. Chem.* **237**, 2652.
- Remmer, H. & Merker, H. J. (1965). *Ann. N.Y. Acad. Sci.* **123**, 79.
- Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J. R., Narasimhulu, S., Cooper, D. Y. & Rosenthal, O. (1966). *Molec. Pharmacol.* **2**, 187.
- Schenkman, J. B., Frey, I., Remmer, H. & Estabrook, R. W. (1967). *Molec. Pharmacol.* **3**, 516.
- Sutherland, E. W., Cou, C. F., Haynes, R. & Olsen, N. S. (1949). *J. biol. Chem.* **180**, 835.