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CHANGES IN CYTOCHROME P-450 IRON SPIN STATE AS A FUNCTION OF CASTRATION, TESTOSTERONE OR ESTROGEN TREATMENT, VIGOROUS EXERCISE, OR STARVATION

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Abstract—3-Methylcholanthrene treatment induced large increases in high-spin P-450 form(s) in intact microsomal membranes from New Zealand White rabbit or C57BL/6N mouse liver but not in rabbit lung, kidney or intestine. Testosterone enhanced the high-spin/low-spin ratio of P-450 in female C57BL/6N liver to the same degree as 3-methylcholanthrene treatment; there was no additive increase in the ratio with treatment of testosterone plus 3-methylcholanthrene. The same effect was seen in ovariectomized females. In the liver of normal or castrated C57BL/6N mice, estradiol-17β pretreatment caused no significant change in the high-spin/low-spin ratio. Vigorous homogenization of the microsomes can decrease the high-spin/low-spin ratio, more so in control than in 3-methylcholanthrene-induced C57BL/6N microsomes. Vigorous exercise (20-min swim) and fasting for 2 days significantly enhanced the high-spin/low-spin ratio in C57BL/6N liver. Temperature studies showed that the low-spin electron paramagnetic resonance signal intensity, as a function of absolute temperature in the range of 16 to 70°K, was consistent with a simple Boltzmann dependence within the Kramers doublet giving rise to the microwave transition. We conclude that, within the limits of measurement, the low-spin state acts like an isolated state and is not in thermal equilibrium with the high-spin form of P-450.

Three fundamentally empirical types of cytochrome P-450 drug-binding difference spectra have been well described and characterized: "Type I", with spectral maxima of about 385 or 395 nm and minima about 420 nm; "Type II", with minima of about 390 or 410 nm and maxima about 427 to 431 nm; and "Reverse Type I", which is almost exactly the mirror image of Type I spectra [1, 2]. Early empirical studies showed apparent discrepancies in the size and shape of these spectra as a function of species [3], strain [4], age and sex [5], tissue [6–8], stress [9], prior treatment *in vivo* [10–13], techniques used during preparation of the microsomes [14, 15], and prior additions to the microsomes *in vitro* [3, 7, 9, 10, 13, 16–21]. Recent spectral binding studies [21–23] and electron paramagnetic resonance (EPR) data [24–26] have made it clear that, the greater the amount of starting high-spin P-450 iron *in vivo*§, the less the peak-to-trough spectral height produced by pure Type I substrates added *in vitro*, and the greater the peak-to-trough spectral height produced by pure Type II or Reverse Type I compounds added *in vitro*. Conversely, the less the amount of starting high-spin P-450 iron in the intact microsomes, the greater the peak-to-trough spectral height produced by pure Type I substrates added *in vitro*, and the lesser the peak-to-trough height produced by pure Type II or Reverse Type I compounds added *in vitro*. It appears that the slow reduction phase of P-450 is controlled by the rate of formation of high-spin P-450 [23].

In this report, changes in the microsomal P-450 spin state of rabbit and mouse are examined. Biological changes in the intact animal—such as castration and sex steroid treatment, vigorous exercise, and starvation—are shown to effect striking changes in the P-450 ferric spin state.

MATERIALS AND METHODS

Materials. Testosterone and estradiol-17β were purchased from the Sigma Chemical Co. (St. Louis, MO). New Zealand White rabbits and C57BL/6N mice were obtained from the Veterinary Resources Branch, National Institutes of Health (Bethesda, MD). Sources for the remainder of the materials have been detailed previously [26].

Treatment of animals and preparation of microsomes. 3-Methylcholanthrene (MC) (200 mg/kg) in corn oil (25 ml/kg) was given intraperitoneally 48 hr before killing; controls received corn oil alone. No paramagnetic differences in microsomes were found between corn oil-treated animals and animals receiving no intraperitoneal injections. All experiments were begun by killing the animals at the same hour

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§ The term "*in vivo*" refers to the intact animal or intact microsomal membranes freshly isolated from the intact animal with the minimal amount of perturbation (excessive salt concentrations, heat, organic solvents, etc.). The term "*in vitro*" refers to subsequent experimental perturbations in the flask or EPR tube.

of day. Microsomal samples were prepared in 0.25 M potassium phosphate buffer, 30% glycerol, pH 7.25, at 4° as previously described [26, 27]. Samples were combined from two (rabbits, male, 1 kg) or twelve (mice, male or female as indicated, 8-weeks-old) animals and were examined by EPR within 1–2 hr of preparation, unless otherwise described. Microsomal protein concentrations ranged between 1 and 2 mg/ml for the P-450 determinations and between 40 and 75 mg/ml for the EPR studies. All experiments were repeated at least once with a second batch of animals to ensure reproducibility.

P-450 content. The CO difference spectral method [28] was used; 91 mM^{-1} cm^{-1} was used as the extinction coefficient for the difference in absorbance between the Soret maximum in the 450-nm region and the 490-nm baseline for the hemoprotein CO complex reduced with dithionite. Protein concentrations were determined with bovine serum albumin as a standard [29].

EPR spectroscopy. Approximately 0.5 ml of each freshly prepared microsomal sample was added to an EPR quartz tube (Varian Associates, Palo Alto, CA), creating a column (0.3 × 6.5 cm). Within a few minutes after the microsomal pellet had been resuspended in the 0.25 M potassium phosphate buffer, 30% glycerol, pH 7.25, and had been added to the EPR tubes, the samples were frozen in liquid nitrogen until EPR examination at either 9.5°K or 77°K. The high-spin EPR measurements were always carried out at 9.5°K, and the low-spin EPR mreasurements were always performed at 77°K in order to avoid power saturation (cf. Ref. 24 for further discussion). A conventional X-band spectrometer with 100 (k)Hz field modulation was used. The 9.5°K temperature was maintained by using the Heli-tran variable-temperature system from the Air Products Co. (Allentown, PA). The 77°K temperature was held constant with the use of a liquid nitrogen dewar. The 9.5°K temperature of the sample was measured by a calibrated germanium resistor (CR 1000, Cryo-Cal, Inc., Riviera Beach, FL) immediately below the sample in the variable temperature dewar. During the measurement of any sample, fluctuation of the 9.5°K temperature by this method varied by less than 0.5°K. Throughout this study, preparation of each sample and EPR measurements were performed always in the same manner. The modulation amplitude, the klystron frequency (approximately 8920 MHz), and the power (about 3 W) were always kept the same, unless otherwise indicated.

Determination of g *value.* The g value determinations were made by calibrating the magnetic field with a proton resonance probe, and the microwave frequency was determined by monitoring a resonance cavity frequency meter which had been calibrated against the 1,1-diphenyl-2-picrylhydrazyl (g = 2.0036) standard [30]. The readings were routinely taken at the peak of the high-spin g_y and at the midpoint of the peak-to-peak amplitude of the low-spin g_y first derivative signals of the EPR absorption. The g_y signal for low spin P-450 ferric iron is referred to in the text as "the $g \cong 2.24$ signal", and the g_y signal for high-spin P-450 ferric iron is referred to as "the $g \cong 8.0$ signal".

RESULTS AND DISCUSSION

P-450 ferric spin state in intact microsomes. It should be emphasized [24–26] that EPR analysis of intact microsomal membranes reflects the *in vivo* P-450 spin state as closely as is technically possible. The

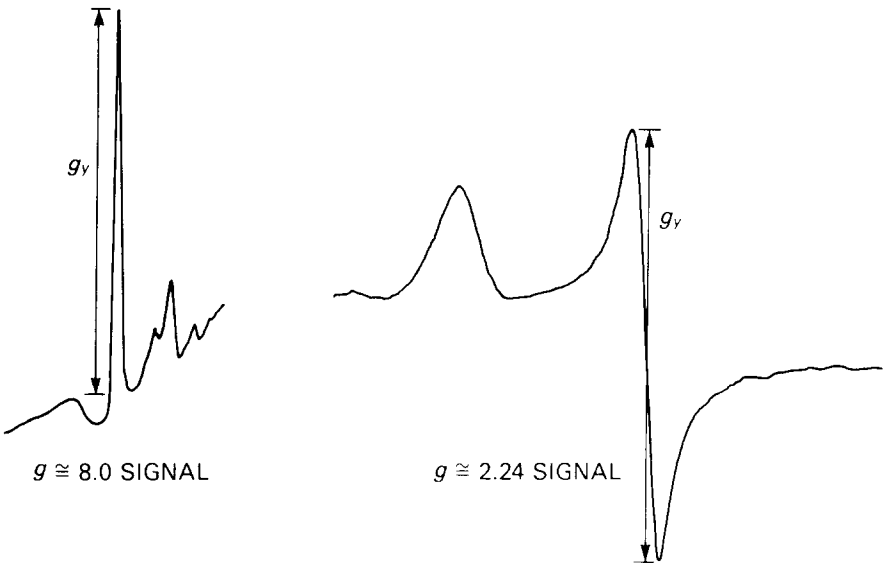

Fig. 1. Typical EPR absorption derivative spectra of high-spin (left) and low-spin (right) P-450 ferric iron in liver microsomes of MC-treated C57BL/6N mice. The field strength (in gauss) is increasing from left to right, in each instance. Left: spectrometer gain was 1250 and temperature was 9.5°K. Right: gain was 2000 and temperature was 77°K. Arrows indicate the height of the g_y high-spin and low-spin signals that were measured. Protein concentration of this sample was 63.2 mg/ml.

Table 1. Ratio of $g \cong 8.0/g \cong 2.24$ and P-450 content in liver, lung, kidney and intestine microsomes from control and MC-treated rabbits

Tissue	Treatment	$g \cong 8.0$ Signal height (Chart units/gain/mg microsomal protein $\times 10^4$)	$g \cong 2.24$ Signal height (Chart units/gain/mg microsomal protein $\times 10^4$)	$g \cong 8.0/g \cong 2.24$ Ratio	Total P-450 content (nmoles/mg protein)
Liver	Control	46	28	1.6*	0.72
	MC	161	20	8.2	1.73
Lung	Control	2.2	4.6	0.48	0.076
	MC	2.5	5.7	0.43	0.135
Kidney	Control	2.5	5.4	0.47	0.11
	MC	2.9	10.4	0.27	0.39
Intestine	Control	0.90	1.7	0.53	0.05
	MC	0.65	1.5	0.44	0.07

* Coefficient of variance (S.D. divided by the mean) in these experiments and in Tables 2, 3 and 4 was always less than 20%. Hence, 1.6 can be regarded at the 95% level of confidence to be significantly different from 8.2; 0.48 and 0.43 not different; 0.47 and 0.27 different; and 0.53 and 0.44 not different.

Table 2. Ratio of $g \cong 8.0/g \cong 2.24$ and P-450 content in C57BL/6N mouse liver microsomes following castration and/or sex steroid treatment

Sex	Castration	Sex steroid treatment	Inducer treatment	$g \cong 8.0$ Signal height (Chart units/gain/mg microsomal protein $\times 10^4$)	$g \cong 2.24$ Signal height (Chart units/gain/mg microsomal protein $\times 10^4$)	$g \cong 8.0/g \cong 2.24$ Ratio	Total P-450 content (nmoles/mg protein)
Female	Sham	None	Control	2.1	5.8	0.36	0.38
			MC	4.6	5.6	0.82	0.71
		Testosterone	Control	5.8	6.8	0.85	0.53
			MC	5.2	5.9	0.88	0.80
	Castrated	None	Control	1.9	4.7	0.40	0.36
			MC	4.5	5.6	0.81	0.56
		Testosterone	Control	6.6	9.0	0.74	0.68
			MC	4.6	6.4	0.71	0.75
Male	Sham	None	Control	1.6	3.9	0.41	0.33
			MC	3.4	4.9	0.70	0.62
		Estrogen	Control	1.0	3.4	0.31	0.24
			MC	4.2	5.3	0.80	0.44
	Castrated	None	Control	2.0	4.1	0.48	0.32
			MC	3.8	5.2	0.73	0.50
		Estrogen	Control	1.6	4.1	0.40	0.25
			MC	7.9	9.3	0.86	0.57

Table 3. Ratio of $g \cong 8.0/g \cong 2.24$ and P-450 content in male C57BL/6N liver microsomes as a function of tissue homogenization

Inducer pretreatment	Homogenization treatment	$g \cong 8.0$ Signal height (Chart units/gain/mg microsomal protein $\times 10^4$)	$g \cong 2.24$ Signal height (Chart units/gain/mg microsomal protein $\times 10^4$)	$g \cong 8.0/g \cong 2.24$ Ratio	Total P-450 content (nmoles/mg protein)
Control	Polytron				
	20 sec	1.7	3.9	0.44	0.30
	2 min	1.3	2.8	0.46	0.30
	4 min	1.1	3.1	0.36	0.30
	Potter–Elvehjem tissue grinder				
	5 strokes	2.1	5.0	0.42*	0.34
	40 strokes	1.3	4.6	0.28*	0.23
MC	Polytron				
	20 sec	3.9	4.8	0.81	0.51
	2 min	4.3	4.7	0.92	0.49
	4 min	3.6	4.3	0.84	0.41
	Potter–Elvehjem tissue grinder				
	5 strokes	4.7	5.1	0.92	0.47
	40 strokes	4.0	5.1	0.79	0.40

* Significantly different ($P < 0.05$).

high-spin/low-spin ratio can be reversibly affected (generally decreased) by treatment of microsomes with organic solvents or detergents [25]; thus, the spin state of purified P-450 preparations might differ from that found in intact unperturbed membranes. Measurement of the high-spin signal at 9.5°K and the low-spin signal at 77°K (Fig. 1) has been shown to give a reasonable approximation of the equilibrium between the high- and low-spin states in intact microsomes, and large differences exist among species [26]. The high-spin $g \cong 8.0$ signal is considerably more labile and sensitive to perturbations than the low-spin $g \cong 2.24$ signal.

It also should be noted that EPR analysis of intact freshly prepared microsomal membranes reflects the summation of spin states and quantities of all contributing forms of P-450. Hence, a minor low-spin form(s) of P-450 might be hidden by a large concentration of a predominating high-spin form(s) of P-450, and vice versa.

Variation among rabbit tissues. The high-spin/low-spin ratio of P-450 was found to be much greater in rabbit liver than in lung, kidney or intestine (Table 1). MC enhanced the total P-450 content more than 2-fold in liver, lung, and kidney, but not intestine. MC increased the high-spin/low-spin ratio in liver

Table 4. Ratio of $g \cong 8.0/g \cong 2.24$ and P-450 content in male C57BL/6N liver microsomes as a function of vigorous exercise or starvation

Inducer pretreatment	Vigorous exercise or starvation	$g \cong 8.0$ Signal height (Chart units/gain/mg microsomal protein $\times 10^4$)	$g \cong 2.24$ Signal height (Chart units/gain/mg microsomal protein $\times 10^4$)	$g \cong 8.0/g \cong 2.24$ Ratio	Total P-450 content (nmoles/mg protein)
Control	None	1.4	4.9	0.29*	0.36
	Vigorous exercise†	1.4	3.8	0.37	0.31
	Fast, 2 days	2.3	5.5	0.41	0.44
	Fast, 4 days	1.1	3.5	0.32	0.32
MC	None	4.3	4.7	0.91‡	0.59
	Vigorous exercise†	6.3	5.6	1.14	0.65
	Fast, 2 days	9.7	8.2	1.18	0.89
	Fast, 4 days	6.3	6.0	1.05	0.62

* The value of 0.29 is significantly different ($P < 0.05$) from 0.37 and 0.41. The value of 0.29 is also significantly lower than the control values listed in Tables 2 and 3, yet the same inbred strain of mouse was used. One possible explanation involves circannual rhythmicity: this experiment was carried out in January and Feburary, whereas the other experiments were performed in late summer and early autumn. At least 2-fold differences in control and MC-inducible aryl hydrocarbon hydroxylase activity have been shown [34] to occur between February and August.

† "Vigorous exercise" denotes a 20-min swim before killing.

‡ The value of 0.91 is significantly different ($P < 0.05$) from 1.14 and 1.18.

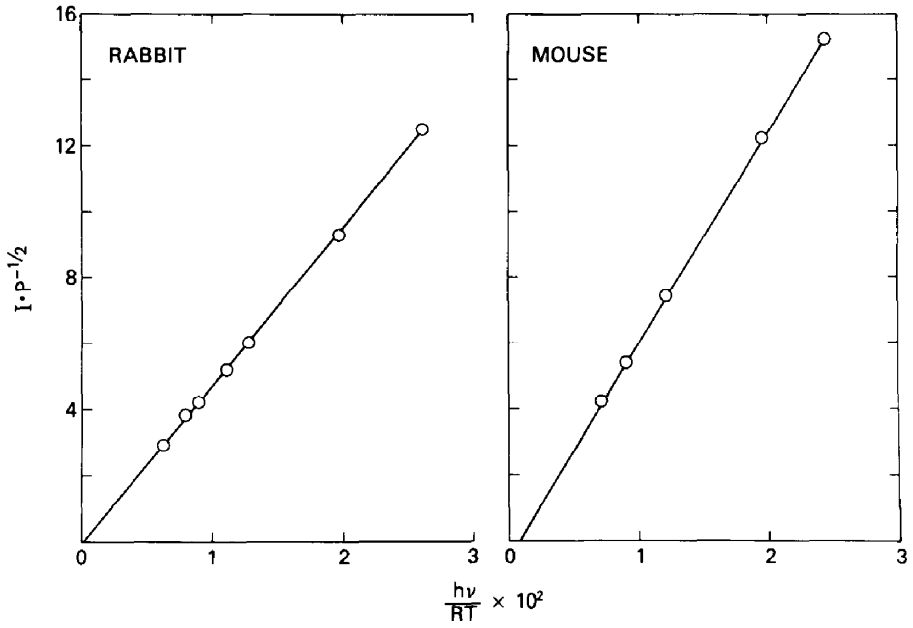

Fig. 2. Intensity of the EPR transitions of the low-spin state of rabbit or mouse hepatic microsomal P-450, as a function of temperature. Key: I, signal height; P, power (mW); h, Planck's constant; v, microwave frequency; k, Boltzmann's constant; and T, degrees Kelvin.

but not in the other three tissues. The predominant high-spin form of P-450—especially in the polycyclic aromatic-treated rabbit—is form 4 [15, 31, 32]. These data (Table 1) suggest that form 4 is markedly inducible by MC in rabbit liver but not in the other three tissues. Such a conclusion is consistent with the finding of Atlas *et al.* [33], who found in several rabbit tissues MC-inducible aryl hydrocarbon hydroxylase activity separable from MC-inducible form 4 (i.e. "P-448").

Castration and testosterone or estradiol-17β treatment in the mouse. The high-spin/low-spin ratio of P-450 in female mouse liver (Table 2) was enhanced by testosterone, whether the female had been ovariectomized or not. MC also increased the high-spin/low-spin ratio, confirming previous data from these laboratories [24–26]. The amount of increase in high-spin P-450 induced by testosterone was not additive with that induced by MC.

In control male mouse liver (Table 2), estrogen produced a slight decrease in the high-spin/low-spin ratio of P-450, but this fall was not significant ($P > 0.05$). MC markedly enhanced the high-spin/low-spin ratio, compared with controls. In MC-treated males, estrogen produced a slight, but not significant ($P > 0.05$), increase in the high-spin/low-spin ratio.

Table 5. Intensity of EPR signal of 1,1-diphenyl-2-picrylhydrazyl standard as a function of temperature

T (°K)	16	23.5	32.5	44.0	56.3	70.0
$IP^{-\frac{1}{2}}$	4.5	4.4	4.3	4.3	4.2	4.1
$\Delta IP^{-\frac{1}{2}}$		0.13	0.21	0.24	0.35	0.46

Homogenization of liver microsomes. As homogenization conditions were made increasingly severe (Table 3), the high-spin/low-spin ratio of P-450 decreased significantly in control, but not significantly in MC-induced, microsomes. These results are consistent with previous findings (discussed in Ref. 26) that control forms of P-450 are more labile than MC-induced forms of P-450, under a variety of *in vitro* conditions.

Vigorous exercise and starvation. Following a 20-min swim (Table 4), vigorous exercise was shown to enhance significantly the high-spin/low-spin ratio of P-450 between 25 and 30% in both control and MC-treated mice. Fasting (no food; water *ad lib.*) caused a significant rise in the high-spin/low-spin ratio at 2 days, but this rise was no longer significant after 4 days of starvation. These data indicate that, during vigorous exercise or fasting, changes in endogenous substrates metabolized by P-450, or in membrane moieties adjacent to P-450, are reflected by differences in the high-spin/low-spin ratio of P-450.

Effects of long-term 77°K storage of microsomes. Samples were stored in liquid nitrogen for as long as 3 months without any detectable changes in the size or shape of the $g \cong 8.0$ and $g \cong 2.24$ signals. No significant change in the high-spin/low-spin ratio of P-450 was seen (results not shown).

Temperature dependence of P-450 spin state between 16 and 70°K. To verify that our measurements of high-spin P-450 at 9.5°K and low-spin P-450 at 77°K provide useful indications of percentages of high- and low-spin iron, we examined the temperature dependence of the spin state in the range from 16°K (lowest temperature at which the low-spin form could be studied) to 70°K. Measurements were made

between 0.01 and 5.0 mW (Fig. 2). Since the intensity of the signal is proportional to the square root of the power, the intensity was normalized as $IP^{-\frac{1}{2}}$. Between 16 and 70°K, there are tables of unsaturation data available for transition intensities versus power. Each data point was taken three times and averaged; coefficients of variance were always less than 1%.

As the temperature was increased, the cavity had to be successively retuned, but the leakage voltage was allowed to drift slightly so that the frequency could be held constant over the range of measurements. To correct for this effect, a sample of 1,1-diphenyl-2-picrylhydrazyl was placed in the cavity outside the dewar as the temperature of the dewar was raised (Table 5). No correction was assumed necessary at 16°K, the temperature at which the cavity was tuned. Table 5 shows the values of the average $IP^{-\frac{1}{2}}$ and the deviations from that value; these corrections were applied to the data in Fig. 2. In the temperature range under consideration, therefore, the intensity of transition within an isolated Kramer's doublet (separation of $h\nu$) is proportional to *i.e.* $^{-h\nu/kT}$, which is approximated by $h\nu/kT$. The excellent fit of the data to the Boltzmann distribution (Fig. 2) is consistent with the absence of a spin-state equilibrium of high- and low-spin P-450 between 16 and 70°K. In other words, these data demonstrate the fundamental soundness of measuring the high-spin P-450 signal at 9.5°K and the low-spin P-450 signal at 77°K.

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REFERENCES

1. H. Remmer, J. Schenkman, R. W. Eastabrook, H. Sasame, J. Gillette, S. Narasimhulu, D. Y. Cooper and O. Rosenthal, *Molec. Pharmac.* **2**, 187 (1966).
2. Y. Imai and R. Sato, *Biochem. biophys. Res. Commun.* **22**, 620 (1966).
3. J. B. Schenkman, D. L. Cinti, S. Orrenius, P. Moldeus and R. Kraschnitz, *Biochemistry* **11**, 4243 (1972).
4. F. M. Goujon, D. W. Nebert and J. E. Gielen, *Molec. Pharmac.* **8**, 667 (1972).
5. G. M. Cohen and G. J. Mannering, *Molec. Pharmac.* **9**, 383 (1974).
6. D. Kupfer and S. Orrenius, *Molec. Pharmac.* **6**, 221 (1970).
7. S. Orrenius, D. Kupfer and L. Ernster, *Fedn Eur. Biochem. Soc. Lett.* **6**, 249 (1970).
8. A. Ellin, S. Orrenius, A. Pilotti and C. G. Swahn, *Archs Biochem. Biophys.* **158**, 597 (1973).
9. C. R. Jefcoate, E. R. Simpson and G. S. Boyd, *Eur. J. Biochem.* **42**, 539 (1974).
10. C. R. E. Jefcoate, J. L. Gaylor and R. L. Calabrese, *Biochemistry* **8**, 3455 (1969).
11. C. R. E. Jefcoate and J. L. Gaylor, *Biochemistry* **8**, 3464 (1969).
12. C. R. E. Jefcoate, R. L. Calabrese and J. L. Gaylor, *Molec. Pharmac.* **6**, 391 (1970).
13. J. B. Schenkman, *Biochemistry* **9**, 2081 (1970).
14. S. Burstein, N. Co, M. Gut, H. Schleyer, D. Y. Cooper and O. Rosenthal, *Biochemistry* **11**, 573 (1972).
15. D. A. Haugen and M. J. Coon, *J. biol. Chem.* **251**, 7929 (1976).
16. J. W. Gorrod, L. G. Disley and D. J. Temple, *Xenobiotica* **1**, 521 (1971).
17. I. Jansson, S. Orrenius, L. Ernster and J. B. Schenkman, *Archs Biochem. Biophys.* **151**, 391 (1972).
18. J. W. Gorrod and D. J. Temple, *Chem. Biol. Interact.* **6**, 203 (1973).
19. J. B. Schenkman, D. L. Cinti, P. W. Moldeus and S. Orrenius, *Drug. Metab. Dispos.* **1**, 111 (1973).
20. M. R. I. Soliman, H. D. Johnson and A. E. Wade, *Drug Metab. Dispos.* **2**, 87 (1974).
21. Y. Yoshida and H. Kumaoka, *J. Biochem., Tokyo* **78**, 455 (1975).
22. K. Kumaki and D. W. Nebert, *Pharmacology* **17**, 262 (1978).
23. W. L. Backes, S. G. Sligar and J. B. Schenkman, *Biochemistry* **21**, 1324 (1982).
24. D. W. Nebert and H. Kon, *J. biol. Chem.* **248**, 169 (1973).
25. D. W. Nebert, J. R. Robinson and H. Kon, *J. biol. Chem.* **248**, 7637 (1973).
26. K. Kumaki, M. Sato, H. Kon and D. W. Nebert, *J. biol. Chem.* **253**, 1048 (1978).
27. A. P. Poland, E. Glover, J. R. Robinson and D. W. Nebert, *J. biol. Chem.* **249**, 5599 (1974).
28. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
29. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
30. D. J. E. Ingram, *Free Radicals as Studied by Electron Spin Resonance.* Butterworths, London (1958).
31. H. H. Liem, U. Muller-Eberhard and E. F. Johnson, *Molec. Pharmac.* **18**, 565 (1980).
32. J. H. Dees, B. S. S. Masters, U. Muller-Eberhard and E. F. Johnson, *Cancer Res.* **42**, 1423 (1982).
33. S. A. Atlas, A. R. Boobis, J. S. Felton, S. S. Thorgeirsson and D. W. Nebert, *J. biol. Chem.* **252**, 4712 (1977).
34. J. R. Robinson, N. Considine and D. W. Nebert, *J. biol. Chem.* **249**, 5851 (1974).