Free Rad. Res. Comms., Vol. 14, No. 1, pp. 9–16
Reprints available directly from the publisher
Photocopying permitted by license only

THE ROLE OF MIXED FUNCTION OXIDASE (MFO) IN THE METABOLISM OF THE SPIN TRAPPING AGENT α-PHENYL-N-TERT-BUTYL-NITRONE (PBN) IN RATS

GUOMAN CHEN, TAMMY M. BRAY[1]†, and EDWARD G. JANZEN[2]

Departments of [1]Nutritional Sciences and [2]Clinical Studies, University of Guelph, Guelph, Ontario, Canada N1G 2W1

and

PAUL B. McCAY[3]

[3]Molecular Toxicology Research Group, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA

(Received July 10, 1990; in revised form August 3, 1990)

It has been previously demonstrated that α-phenyl-N-tert-butyl-nitrone (PBN), one of the most widely used free radical trapping agents, is rapidly absorbed, evenly distributed among a wide range of tissues, and metabolized to form one metabolite. The objective of this study was to determine if the distribution and metabolism of PBN can be affected by inducers or inhibitors of the microsomal mixed function oxidase (MFO) system. Rats were pretreated with MFO inducers (phenobarbital and 3-methylcholanthrene) or MFO inhibitors (metyrapone and piperonyl butoxide) before ^{14}C-PBN was injected (i.p.) The concentrations of ^{14}C-PBN and its metabolite were measured in plasma, urine, liver, lung and kidney 2 hours after injection. The results indicated that when MFO was induced, the concentration of ^{14}C-PBN metabolite was significantly reduced in all tissues measured. The maximum concentration of PBN parent compound in the tissues where MFO was induced was 50% of that found in saline controls. Manipulation of tissue MFO levels with inducers and inhibitors altered the ratio of ^{14}C-PBN parent compound to the PBN-metabolite. When ^{14}C-PBN was incubated with rat liver microsomes at 37°C in the presence of NADPH, the rate of metabolism was 1,752 dpm of ^{14}C-PBN-metabolite formed/nmole P-450/min. Inactivation of MFO by heat (80°C for 1 min) or deletion of NADPH diminished the formation of PBN metabolite *in vitro*. It is concluded that the MFO system may be responsible for the metabolism of PBN. Tissue concentrations of PBN can be affected by drugs or toxins which are inducers or inhibitors of MFO.

KEY WORDS: α-phenyl-N-tert-butyl-nitrone (PBN), PBN-metabolite, phenobarbital, 3-methylcholanthrene, metyrapone, piperonyl butoxide, mixed function oxidase (MFO), MFO inducers and MFO inhibitor.

INTRODUCTION

We reported that PBN, one of the most widely used spin trapping agents, is rapidly absorbed, evenly distributed among a wide range of tissues and excreted slowly when

†Correspondence: Dr. T.M. Bray, Department of Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada, N1G 2W1, Tel. (519) 824-4120, Ext. 3752, Fax. (519)-763-5902

it is injected intraperitoneally in rats. There was only one form of PBN-metabolite excreted in the urine.[1] In our pharmacokinetic study of PBN, the data successfully fitted a two-compartment pharmacokinetic model with a first order absorption step. The PBN concentration in plasma peaked as early as 15 min while in tissues it reached a maximum concentration at 30 min after administration.[1,2]

The use of spin trapping agents to investigate free radical processes in biological systems has been focused primarily on the detection and identification of the radicals trapped after the biological system has been exposed to foreign chemicals or toxins. The efficiency of spin trapping is proportional to the concentration of the spin trapping agent maintained in the specific tissue. It has been shown that PBN affects the mixed function oxidase (MFO) system in rat hepatocytes and liver microsomes,[3] but the amount of PBN which can be metabolized by MFO in various tissues is still unknown. Very little information is available concerning the effect of foreign chemicals on PBN metabolism *in vivo*. A better understanding of the effect of pretreatment of drug or toxin on the rate of PBN metabolism is essential for determining the optimum conditions that can be used in designing experimental protocols for trapping free radicals *in vivo*.

The objective of this study was to determine if PBN can be metabolized by the MFO system, and if the tissue distribution and metabolism of PBN can be affected by the inducers (phenobarbital and 3-methylcholanthrene) or inhibitors (metyraprone and piperonyl butoxide)[4–8] of the microsomal MFO system.

MATERIALS AND METHODS

Materials

PBN, metyrapone and 3-methylcholanthrene were obtained from Sigma (St. Louis, MO). The α-phenyl-α-[^{14}C]-N-tert-butyl nitrone was custom synthesized by Amersham (Arlington Heights, IL). The specific activity of ^{14}C-PBN was 2.7 mci/mmol with a purity of 98.15%. Phenobarbital was obtained from Ontario Veterinary College, University of Guelph. Piperonyl butoxide was purchased from ICN Biochemicals Inc. (Plainview, NY). Methanol (HPLC grade) was obtained from Fisher Scientific (Toronto, Ontario). Scintillation fluid, Aquasol-2, was purchased from New England Nuclear (Boston, MA). NADPH was purchased from Boehringer Mannheim Canada (Dorval, Que).

Animal Care

Male Wistar rats (Charles River, Montreal, Quebec) weighing 200–250 g were housed individually in suspended stainless steel metabolic cages in a temperature and humidity controlled room with light from 0800 to 2000 h. Rats were fed ad libitum with a chow diet (Purina, St. Louis, Mo) and had free access to water.

Experimental Design

Rats were divided into 5 groups. Prior to PBN administration (75 mg containing 4 μCi/kg body weright, i.p.), rats were injected i.p. with the following compounds: (1) saline, (2) phenobarbital (PB), dissolved in saline and given at a dose of 80 mg/kg

body weight once daily for 3 days, (3) 3-methylcholanthrene (3-MC), dissolved in corn oil and given at a dose of 100 mg/kg body weight once daily for 3 days, (4) metyrapone (MP), dissolved in propylene glycol and given at a dose of 50 mg/kg body weight 2 h before PBN administration, (5) piperonyl butoxide (BT), dissolved in corn oil and injected at a dose of 50 mg/kg body weight 24 h, 12 h and 6 h prior to PBN administration.

Based on the previous data on tissue distribution and metabolism of PBN,[1] the possible changes of the ratio of PBN to its metabolite present in urine and tissues due to pretreatment of MFO inducer or inhibitor can be most likely detected 2 h after administration of PBN. Thus, urine from individual rat was collected for 2 h post-PBN administration at which time the rats were sacrificed. Blood was withdrawn immediately from the left ventricle into heparinized tubes. Approximately 30 ml of ice-cold isotonic saline solution was perfused *in situ* through the right side of the heart to remove blood from lung and liver. Liver, lung and kidney were immediately removed, weighed, and stored at −80°C.

Sample Preparation and Analysis of PBN and PBN-Metabolite

Methanol (3.5 ml) was added to 1.5 ml of plasma or urine. The mixture was vortexed and centrifuged at 20,000 × g for 60 min. The supernatant fraction was collected and was used for HPLC injection. Tissue samples (1 gm) were homogenizaed in 1 ml of methanol in water (70/30, v/v) and then centrifuged at 20,000 × g for 20 min. The supernatant fraction, after addition of 0.2 ml of trichloroacetic acid (10%), was again centrifuged at 20,000 × g for 20 min. The supernatant fraction was collected and was used for HPLC injection.

Reverse-phase high performance liquid chromatography (HPLC) and liquid scintillation spectroscopy were used to identify and quantitate the PBN and PBN-metabolite in urine, plasma and tissue samples.[1] The HPLC was equipped with a dual piston pump (Varian 2010), a variable UV absorbance detector (Varian 2050) set at 289 nm, and a μ Bondapac™ C_{18} column (4.6 mm × 25 cm, Waters Division of Millipore). The mobile phase was a mixture of methanol/water (70/30, v/v). The flow rate was 1.0 ml/min, and the UV spectrum was recorded (Linear 1200) at a chart speed of 1.0 cm/min. Fractions (1.5 ml each) from the HPLC eluate were collected and counted for radioactivity in 10 ml of Aquasol-2.

To verify the efficacy of the inducers and inhibitors of MFO, tissue microsomal cytochrome P-450 contents were measured after drug pretreatment. Microsomes were isolated according to the method of Poyer *et al.*,[9] with minor modifications. Tissues (liver, kidney, and lung) were immediately removed after rats were sacrificed by decapitation. Tissues (1 g of wet tissue per 5 ml of buffer) were homogenized in 0.01 M Na phosphate buffer with 0.15 M KCl, pH 7.4 (microsomal buffer). The homogenate was centrifuged at 10,000 × g for 15 min. The supernatant fraction was collected and centrifuged at 105,000 × g for 60 min. The pellet was washed 3 times with the microsomal buffer and resuspended in the buffer. Microsomal cytochrome P-450 content was determined from the reduced CO-complexed difference spectrum,[10] using an extinction coefficient of 91 mM^{-1} cm^{-1}. The microsomal protein was analyzed by the method of Lowry *et al.*[11]

Since the MFO inducer, phenobarbital, may increase the metabolism of PBN and decrease the concentration of PBN in tissues, the rate of metabolism of PBN after phenobarbital pretreatment was investigated. Prior to the injection of PBN, rats were

given PB (80 mg/kg body weight, i.p.) for 3 days. Rats were sacrificed at 15, 30, 60 and 120 min after PBN administration. Preparation of tissue samples for quantitation of PBN was as previously described. The PBN concentration was calculated by comparison of sample peak height with that of a known concentration of a standard PBN according to UV chromatogram.

To test if PBN can be metabolized by the MFO system *in vitro*, ^{14}C-PBN (1.0 mM, 9.58 μCi/mmol) was incubated at 37°C with 3.4 mg of microsomal protein (0.96 nmol P-450/mg protein) and 3 mM NADPH in total voluume of 1.0 ml of the microsomal buffer. The control contained the same constituents but the microsomes had been heated at 80°C for 1 min. After incubation, 0.7 ml methanol was added into 0.3 ml of the incubation mixture, vortexed for 1 min and centrifuged at 20,000 × g for 20 min. The supernatant fraction was collected and was used for HPLC injection. The fractions (1.5 ml each) from the HPLC eluate were collected and counted for radioactivity in 10 ml Aquasol-2.

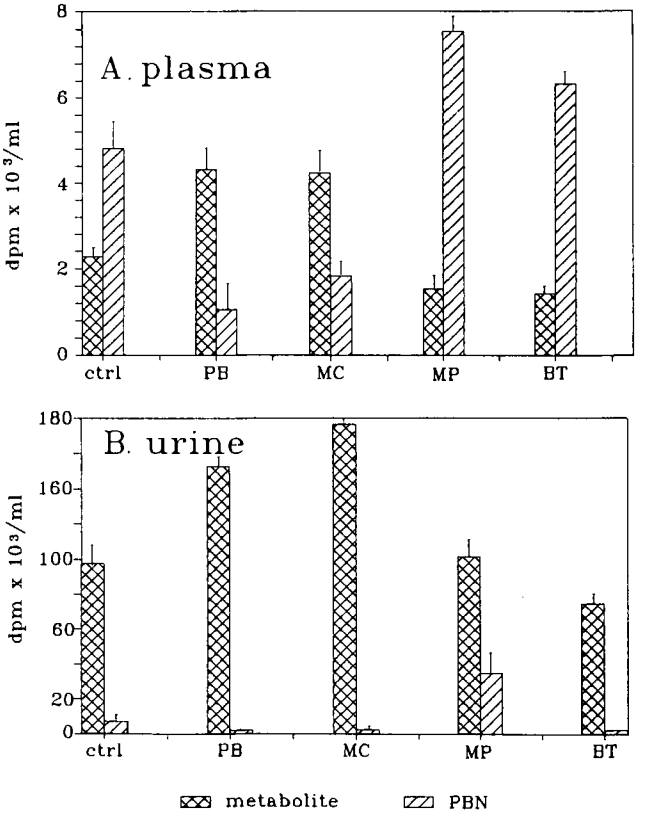

FIGURE 1 Effect of pretreatment of MFO inducers and inhibitors on the concentrations of PBN and PBN-metabolite in plasma and urine. Rats (n = 4) were pretreated with saline (ctrl), phenobarbital (PB), 3-methylcholanthrene (MC), metyrapone (MP) or piperonyl butoxide (BT) prior to ^{14}C-PBN injection. Urine was collected for 2 h post-PBN administration. Blood was taken at 2 h. ^{14}C-PBN and ^{14}C-PBN metabolite were separated by HPLC and counted for radioactivity.

RESULTS AND DISCUSSION

The radioactivities of ^{14}C-PBN and ^{14}C-PBN-metabolite in plasma (Figure 1A) and urine (Figure 1B) of rats with pretreatment of MFO inducers and inhibitors are compared to that of the control group. In the plasma of control rats, the radioactivity of PBN-metabolite is approximately one half that of the parent PBN. When compared to the control group, rats pretreated with known MFO inducers, PB or 3MC, the radioactivity of PBN-metabolite increased while the parent PBN decreased significantly. In contrast, when rats were pretreated with known MFO inhibitors, mety-

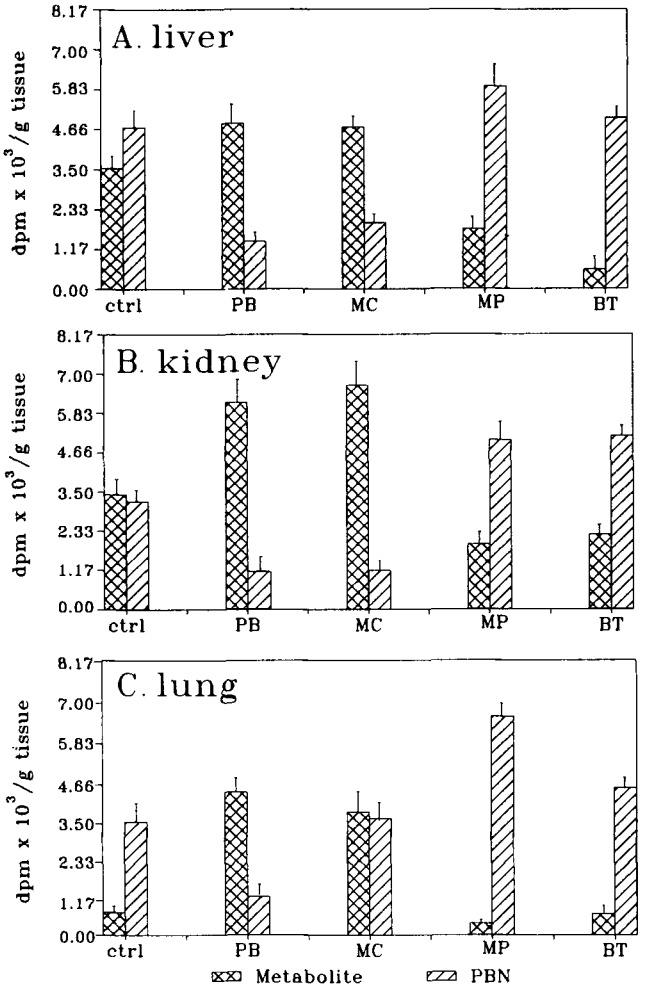

FIGURE 2 Effects of pretreatment of MFO inducers and inhibitors on the concentrations of PBN and PBN-metabolite in liver, kidney and lung. Rats (n = 4) were pretreated with saline (ctrl), phenobarbital (PB), 3-methylcholanthrene (MC), metyrapone (MP) or piperonyl butoxide (BT) prior to ^{14}C-PBN injection. Tissues were removed 2 h after ^{14}C-PBN administration. ^{14}C-PBN and ^{14}C-PBN metabolite were separated by HPLC and counted for radioactivity.

rapone or piperonyl butoxide, the radioactivity of PBN-metabolite in the plasma is lower and the parent compound PBN in the plasma is significantly higher than that of the control.

Two hours afer ^{14}C-PBN administration, 90% of radioactivity in the urine of control rats is in the form of PBN-metabolite (Figure 1B). Pretreatment with PB or 3MC increases the amount of urinary PBN-metabolite excreted. However, neither of the MFO inhibitors significantly reduced the total amount of PBN-metabolite excreted in the urine. It is possible that the PBN-metabolite is preferentially excreted in the urine since the PBN-metabolite is more water soluble than the parent compound.

The effects of pretreatment with MFO inducers and inhibitors on the tissue concentrations of ^{14}C-PBN and ^{14}C-PBN-metabolite are illustrated in Figure 2A–C. The

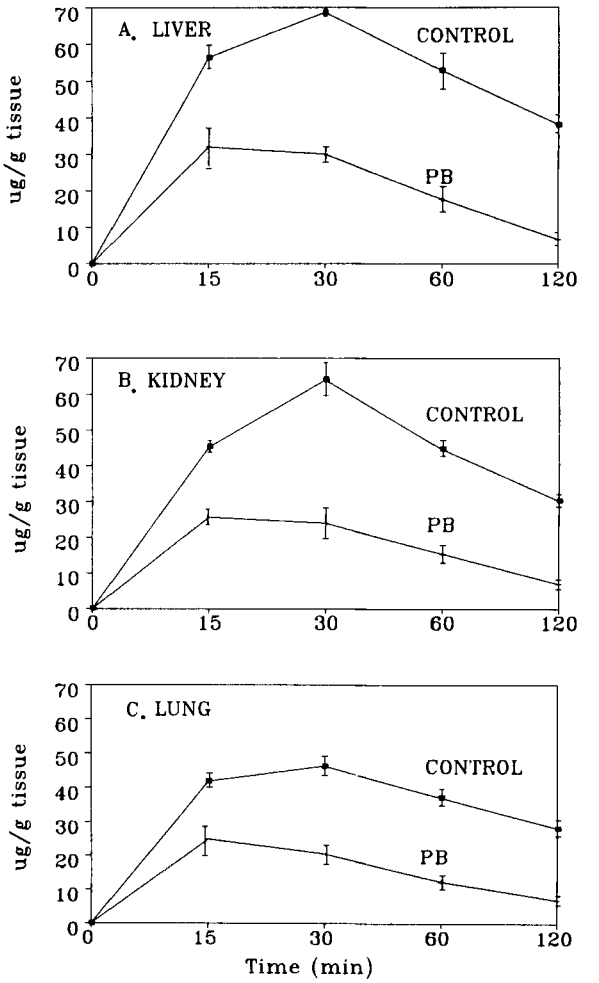

FIGURE 3 Effects of pretreatment with phenobarbital on the tissue concentration of PBN at different time intervals. Prior to the injection of PBN, rats (n = 4) were given phenobarbital daily for 3 days. Tissue concentrations of PBN were analysed with HPLC method as described in *Materials and Methods* section.

ratio of the concentration of PBN-metabolite/PBN in the tissue changes consistently with the hypothesis that MFO affects the metabolism of PBN. The concentration of ^{14}C-PBN metabolite increases when rats were pretreated with inducers, whereas the concentration of parent compound, ^{14}C-PBN increases when rats were pretreated with inhibitors. The effects of MFO inducers and inhibitors were more pronounced in liver and kidney than in lung.

To obtain the optimal tissue concentration of PBN for spin trapping, the effects of PB pretreatment on the concentration of PBN in liver, kidney and lung at different time intervals after administration of PBN were determined (Figure 3). In all tissues measured, PB pretreatment reduced the total concentration of PBN in the tissues, and the peak concentration of PBN was approximately 15 min earlier than that of the control.

To assure that the MFO enzyme system in the tissues was induced or inhibited, tissue concentrations of cytochrome P-450 were measured after treatment of inducers or inhibitors without the administration of PBN (Table 1). Phenobarbital and 3MC significantly induced cytochrome P-450 in the liver, kidney and lung. BT significantly reduced cytochrome P-450 content in the liver. Since the concentration of cytochrome P-450 is not detectable in the kidney and lung in control group, no effect of the inhibitors was measured.

Table 2 indicates that PBN was metabolized by NADPH-dependent MFO system *in vitro*. Deletion of NADPH from the incubation medium or destruction of microsomal enzymes by heat diminished the conversion of PBN to PBN metabolite.

TABLE I
Effects of pretreatment of MFO inducers and inhibitors on the tissue concentration of cytochrome P-450[1]

	liver	kidney	lung
MP	0.561 ± 0.11*	ND**	ND
BT	0.464 ± 0.02	ND	ND
Control	0.636 ± 0.01	ND	ND
PB	1.065 ± 0.05	0.062 ± 0.025	0.032 ± 0.01
3-MC	0.990 ± 0.11	0.027 ± 0.001	0.020 ± 0.01

[1]units = nmole/mg microsomal protein
*Mean value ± SEM, 3 animals in each group.
**Non-detectable

TABLE II
In vitro microsomal metabolism of ^{14}C-PBN

Incubation Condition	Rate of PBN metabolism[1] (dpm of ^{14}C-PBN metabolite formed/ nmole P-450/min)
^{14}C-PBN with NADPH + fresh microsomes	1,752*[a] ± 85
^{14}C-PBN without NADPH + fresh microsomes	513[b] ± 12
^{14}C-PBN with NADPH + boiled microsomes*	350[b] ± 46

*Liver microsomes were boiled for 1 min
[1]Mean ± SEM. 3 animals in each group.
^{14}C-PBN-metabolite in incubation medium was separated by HPLC and the radioactivity was counted by scintillation counter. Values with different superscript letters ([a,b]) are significantly different ($P < 0.05$).

This work clearly demonstrates that PBN can be metabolized by the microsomal MFO system. *In vivo* manipulation of MFO enzyme activities with inducers or inhibitors can alter the rate of PBN metabolism. PBN is metabolized *in vitro* by a NADPH-dependent microsomal system. Although the structure of the PBN-metabolite is still under investigation, PBN-metabolite can be quantified by the combined techniques of HPLC and liquid scintillation counting. PBN is often used in conjunction with or after administration of toxins or drugs. If the toxins or drugs tested are known MFO inducers, it is advisable to isolate the radical-PBN adduct approximately 15 min after administration of PBN.

Acknowledgements

This research is supported by NSERC grants to T.M. Bray and E.G. Janzen and NIH grant (GM 36512) to P.B. McCay. The postdoctoral fellowship to G. Chen is partially supported by the College of Biological Science, University of Guelph.

References

1. G. Chen, T.M. Bray, E.G. Janzen and P.B. McCay (1990) Excretion, metabolism and tissue distribution of a spin trapping agent, α-phenyl-N-tert-butyl-nitrone (PBN) in rats. *Free Radical Research Communications*, **9**, 317–323.
2. G. Chen, M.T. Griffin, J.L. Poyer, P.B. McCay and D.W.A. Bourne (in press) HPLC procedures for the pharmacokinetic study of the spin trapping agent, α-phenyl-N-tert-butyl-nitrone (PBN). *Free Radical Biology and Medicine*.
3. E. Albano, K.H. Cheeseman, A. Tomasi, R. Carini, M.U. Dianzani and T.F. Slater (1986) Effect of spin traps in isolated rat hepatocytes and liver microsomes. *Biochemical Pharmacology*, **35**, 3955–3960.
4. T.M. Bray and J.R. Carlson (1979) Role of mixed-function oxidase in 3-methylindole-induced acute pulmonary edema in goats. *American Journal of Veterinary Research*, **40**, 1268–1272.
5. T. Noguchi, K.L. Fong, E.K. Lai, S.S. Alexander, M. Margaret King, L. Olson, J.L. Poyer and P.B. McCay (1982) Specificity of a phenobarbital-induced cytochrome P-450 for metabolism of carbon tetrachloride to the trichloromethyl radical. *Biochemical Pharmacology*, **31**, 615–624.
6. Y. Masuda and M. Yasoshima (1988) Loss of 3-methylcholanthrene-inducible form of cytochrome P-450 in liver microsomes following adminstration of carbon disulfide in C57BL/6 Cr mice. *Biochemical Pharmacology*, **37**, 2363–2371.
7. M. Kung, S.W. Kooh, W. Paek and D. Fraser (1987) Differential behaviour of 25-hydroxyvitamin D_3 24-hydroxylase and 1-hydroxylase in response to protein synthesis inhibitors and cytochrome P-450 inhibitors. *Canadian Journal of Physiology and Pharmacology*, **66**, 586–591.
8. M. Numazawa and S. Satoh (1988) Kinetic studies of inhibition of estradiol 2- and 16-hydroxylases in rat liver microsomes with various cytochrome P-450 inhibitors. *Journal of Steroid Biochem.*, **29**, 221–226.
9. J.L. Poyer, R.A. Floyd, P.B. McCay, E.G. Janzen and E.R. Davis (1978) Spin-trapping of the trichloromethyl radical produced during enzymic NADPH oxidation in the presence of carbon tetrachloride or bromotrichloromethane. *Biochimica et Biophysica Acta*, **539**, 402–409.
10. T. Omura and R. Sato (1964) The carbon monoxide-binding pigment of liver microsomes. Solubilization, purification and properties. *Journal of Biological Chemistry*, **239**, 2379–2385.
11. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.