Table 2. Alteration in testosterone hydroxylation activity of rat hepatic microsomes

Microsomes	Testosterone hydroxylation activity							
	6α-OH	15α-OH	7α-OH	6β-OH	16α-OH	16β-OH	2α-OH	2β-OH
UT	—	—	—	2.39 ± 0.26	1.51 ± 0.04	0.08 ± 0.04	1.04 ± 0.26	0.31 ± 0.01
MS	—	—	—	3.52 ± 0.13*	2.29 ± 0.52	0.54 ± 0.01†	0.93 ± 0.28	0.49 ± 0.01†
PB	—	—	—	5.93 ± 0.83*	2.52 ± 0.13†	1.46 ± 0.17†	0.50 ± 0.04	0.95 ± 0.09*

Measurements were made from two to four different preparations of microsomes in duplicate, and the values are expressed as mean ± SD in nmol of product/min/mg of microsomal protein. Values less than 0.08 nmol/min/mg protein are expressed as "—".

The abbreviations refer to the position of testosterone hydroxylation.

UT, untreated; MS, muscone-treated; PB, phenobarbital-treated.

* $P < 0.05$, † $P < 0.01$.

that P450 PB-4 and PB-5 are the major PB-inducible forms of P450 and P450, PB-1 and PB-2 were weakly inducted with PB. We have found in agreement with other authors that none of these forms were induced in the liver of rats treated with MC (MC treatment data not shown). Our results with muscone agreed with the PB-inducible pattern [4]. In addition acetone- or alcohol-inducible form (P450 IIE1) of P450 did not induce with muscone treatment [6]. The testosterone hydroxylation activity of both muscone- and PB-treated hepatic microsomes is shown in Table 2. The 2β- and 6β-hydroxylation activity were increased 1.6- and 1.5-fold with muscone, and 3.1- and 2.5-fold with PB, respectively. The 16β-hydroxylation activity of testosterone was strongly induced 6.8- and 18.3-fold with muscone and PB, respectively. On the contrary, testosterone 2α-hydroxylation activity was suppressed by muscone and PB treatment. Our results are in accordance with those of Imaoka *et al.* [4].

These results suggest that the induction type of muscone treatment is very similar to that induced by PB, mainly affecting PB-4/5 but with lesser magnitude than PB. This data supports and extends our previously published findings with muscone [1, 2]. Therefore, care must be taken in the decrease of therapeutic effects when is given with muscone in the clinical use.

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REFERENCES

1. Peng R, Zhu X-Y and Yang S, *Biochem Pharmacol* **35**: 1391, 1986.
2. Tanaka E, Kurata N, Kohno M, Yoshida T and Kuroiwa Y, *Biochem Pharmacol* **36**: 4263, 1987.
3. Imaoka S, Terano Y and Funae Y, *J Biochem* **104**: 481, 1988.
4. Imaoka S, Terano Y and Funae Y, *J Biochem* **105**: 939, 1989.
5. Imaoka S, Terano Y and Funae Y, *Arch Biochem Biophys* **278**: 168, 1990.
6. Funae Y, Imaoka S and Shimojo N, *Biochem Int* **16**: 503, 1988.
7. Imaoka S, Terano Y and Funae Y, *Biochim Biophys Acta* **916**: 358, 1987.
8. Bandiera S, Ryan DE, Levin W and Thomas PE, *Arch Biochem Biophys* **248**: 658, 1986.
9. Funae Y and Imaoka S, *Biochim Biophys Acta* **926**: 349, 1987.
10. Imaoka S, Kamataki T and Funae Y, *J Biochem* **102**: 843, 1987.
11. Nebert DW, Nelson DR, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R and Waterman MR, *DNA* **1**: 8, 1989.

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Biochemical Pharmacology, Vol. 41, No. 3, pp. 473–476, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Determination of the *in vivo* antigen–antibody affinity constant from the redistribution of desipramine in rats following administration of a desipramine-specific monoclonal antibody

(*Received* 12 *June* 1990; *accepted* 27 *September* 1990)

Although there are many *in vitro* procedures for determining the affinity of antibodies for antigen, little attention has been given to the possibility that the effective binding constant *in vivo* may be amenable to calculation. This communication describes such a procedure and illustrates its use by analysis of the redistribution of desipramine (DMI) in rats following administration of a monoclonal antibody specific for this tricyclic antidepressant (anti-TCA).

Materials and Methods

Animal studies. The animal studies have been described

in detail elsewhere [1]. Briefly, male Holtzman rats (150–350 g) were anaesthetized and cannulae were inserted into the femoral veins. [^{3}H]Desipramine hydrochloride was administered intravenously as a bolus, supplemented on some occasions with unlabelled DMI, to give administered doses in different groups of 0.0001, 0.001, 0.01 and 1 mg (N = 5 or 6 per dose). Fifteen minutes later, anti-TCA (2, 10 or 50 mg) was infused over 2 or 10 min. Blood samples for determination of plasma DMI concentration were collected between 0.2 and 14 min thereafter. The molar ratio of anti-TCA binding sites to DMI administered ranged between 0.004 to 210. DMI concentrations were determined either by liquid scintillation counting or by HPLC [1]. There was no appreciable metabolism of the drug in the interval between administration of the dose and the time of blood collection.

Desipramine-specific antibody. The anti-TCA was a murine monoclonal antibody (IgG1), for which the interaction with DMI is governed by an intrinsic association constant, K_{Ab}, of 0.3 nM^{-1} [1] when measured by the method of Muller [2]. This binding constant was confirmed by a quantitative ELISA technique [3], which yielded a value of 0.15 (±0.02) nM^{-1}. The purity of the anti-TCA was 74% [1].

Determination of the in vivo *association constant.* After administration of a dose, D, of DMI (antigen), Ag, the volume of distribution, V_d, is calculated from the plasma concentration of DMI measured immediately prior to antibody administration, $[Ag]_{preAb}$, according to

$$V_d = D/[Ag]_{preAb}. \quad (1)$$

Following antibody administration, there is a change in the plasma concentration of the drug to a new value $[Ag]_{tot}$, which includes unchanged DMI bound to antibody and α_1-acid glycoprotein (AAG), as well as free (unbound) drug. Subject to the assumption that the IgG is confined to the plasma volume (V_p), the dose of administered antigen can be expressed as

$$D = [Ag](V_d - V_p) + V_p[Ag]_{tot}, \quad (2)$$

where [Ag] is the concentration of drug not bound to antibody. The term $V_p[Ag]_{tot}$ expresses the amount of drug in the plasma volume. $[Ag](V_d - V_p)$ describes the balance of administered drug in terms of the remainder of the distribution volume and the consequent concentration of drug required for mass conservation. Rearrangement of Eqn 2 then gives

$$[Ag] = (D - V_p[Ag]_{tot})/V_d - V_p, \quad (3)$$

which allows calculation of the combined concentrations of free drug and its complex with AAG. [Ag] can also be expressed as

$$[Ag] = [Ag]_u + K_{AAG}[Ag]_u[AAG]_u = [Ag_u\{1 + K_{AAG}[AAG]/(1 + K_{AAG}[Ag]_u)\}, \quad (4)$$

where K_{AAG} is the binding constant for the interaction of DMI with the single site on AAG, present at total concentration [AAG]. $[AAG]_u$, the corresponding free concentration, has been eliminated by expressing [AAG] as the sum of $[AAG]_u$ and the concentration of complex, i.e. by noting that $[AAG] = [AAG]_u(1 + K_{AAG}[Ag]_u)$.

The unbound concentration of DMI, $[Ag]_u$, then follows from the expression

$$[Ag]_u = \frac{-\{1 + (K_{AAG}[AAG] - [Ag])\} + \{1 + K_{AAG}([AAG] - [Ag])\}^2 + 4K_{AAG}[Ag]^{1/2}}{2K_{AAG}} \quad (5)$$

which is the solution of the quadratic obtained by expressing [Ag] as the sum of $[Ag]_u$ and the concentration of drug complexed with AAG.

The binding function, ν, was then defined as

$$\nu = ([Ag]_{tot} - [Ag])/[Ab], \quad (6)$$

where [Ab] is the total concentration of antibody (amount administered divided by the plasma volume). The resulting $(\nu,[Ag]_u)$ data were then used to evaluate K_{Ab} in plasma *in vivo* by nonlinear regression analysis [4].

Selection of parameter values. The volume accessible to anti-TCA was equated with the plasma volume, 0.04 L/g [5], because of the brief period (0.2–14 min) that elapsed between administration of the antibody and the measurement of the plasma concentration of DMI. Allowance for the interaction of DMI with AAG was based on a concentration of 7.5 μM for the protein in rat plasma [6] and an association constant (K_{AAG}) of 0.13 μM^{-1} for the interaction [7]. The molecular weight of IgG was taken as 150,000 Daltons.

Results and Discussion

Before using this method to determine the binding constant for the anti-TCA interaction *in vivo*, it was necessary to demonstrate that V_d is dose-dependent. Relevant data, summarized in Fig. 1, show no obvious dependence of V_d (expressed on a kg basis) on administered dose, similarly normalized. The mean (±SD) value of 3.6 ± 0.9 L/kg is indicated by the solid line.

The Scatchard plot [8] of the binding data exhibited no obvious departure from linearity (Fig. 2). Linear regression analysis of the untransformed $(\nu,[Ag]_u)$ data yielded values of 4.7 ± 0.4 μM^{-1} for K_{Ab} and 1.7 ± 0.1 for the number of binding sites. The number of binding sites is smaller than the theoretical value of 2 for IgG. This disparity could reflect overestimation of the antibody concentration, failure of the antibody to be confined to the plasma volume, or some catabolism of the antibody before measurement of the plasma DMI concentration. The latter two explanations seem unlikely in view of the very short time that elapsed between anti-TCA administration and measurement of the plasma concentration of DMI.

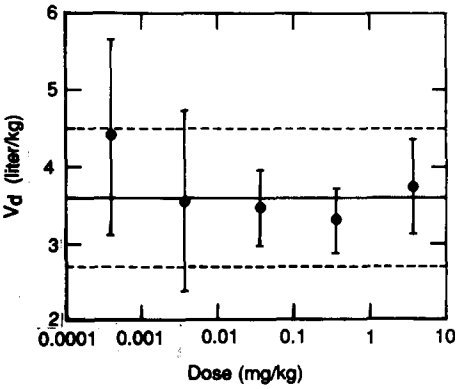

Fig. 1. Test for dose-independence of the distribution volume of DMI (V_d), normalized to a kg basis in rats. Experimental points denote the means (±SD) for five or six animals. The line is based on a dose-independent V_d of 3.6 L/kg.

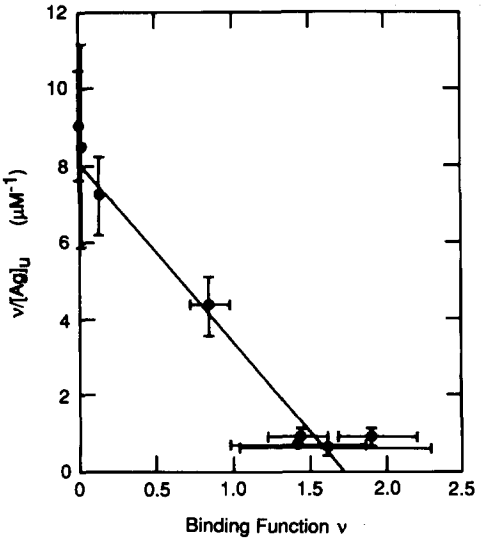

Fig. 2. Scatchard plot of binding data for the interaction of desipramine with its specific monoclonal antibody *in vivo*. Experimental points denote the means (±SD) for five or six animals. The line is based on nonlinear regression analysis of the untransformed (v,$[Ag]_u$) data.

Uncertainty in the number of binding sites does not affect the validity of the estimate of K_{Ab}. The K_{Ab} of 4.7 μM^{-1} obtained *in vivo* is substantially smaller than the values of 0.3 nM^{-1} [1] and 0.15 nM^{-1} (this study) found by two independent *in vitro* methods [2, 3]. This difference cannot be attributed to the binding of DMI to additional acceptors or receptors outside the plasma volume as this has been taken into account in the determination of the V_d of DMI. That this difference is due to a biological effect, not related to the method of calculating the *in vivo* K_{ab}, is supported by the findings of a separate unpublished study. Five rats (140–160 g) were given a bolus dose of 4.5 mg DMI intravenously, 5 min before the termination of a 45 min infusion of the same anti-TCA. The molar ratio of anti-TCA binding sites to DMI was 0.55. Blood was taken 15 min after the DMI dose. The unbound plasma concentration of DMI was measured by equilibrium dialysis [9], total DMI concentration by HPLC [10] and the concentration of anti-TCA antibody by radial immunodiffusion [11]. The K_{ab} for this group was $0.48 \pm 0.22\ \mu M^{-1}$, even lower than the K_{ab} calculated from Eqn 6. The two values can probably be regarded as similar because of the errors involved in each of the experimental measurements required to obtain the value of K_{Ab}, particularly those in the equilibrium dialysis method.

The difference between the *in vivo* and *in vitro* estimates of K_{Ab} presumably indicates the presence of plasma ligands for which the anti-TCA exhibits affinity. From the thermodynamic viewpoint, it is immaterial whether the diminished binding constant *in vivo* reflects the binding of the putative ligand(s) to the DMI binding site or elsewhere. The fact that the K_{Ab} obtained from *in vivo* measurements may be a composite quantity because of several interactions should not be regarded as a criticism of the method. Indeed, from the immunotherapeutic viewpoint, the *in vivo* calculations yield the K_{Ab} with greater clinical relevance. Furthermore, the value of K_{Ab} obtained *in vivo* is still more pertinent to clinical situations even if the disparity between equilibrium constants were to reflect pseudoequilibrium in the *in vivo* experiments. The important factor in any immunotherapeutic treatment of TCA overdose is the short-term, rather than the ultimate binding of the tricyclic antidepressant by the anti-TCA.

In summary, quantitative expressions have been derived to determine the affinity constant for the *in vivo* interaction of an antigen with its elicited monoclonal antibody by analysing the redistribution of antigen following antibody administration. Using this method, the intrinsic binding constant for the interaction of subtoxic doses of DMI in rats with anti-TCA was found to be about two orders of magnitude less than the value obtained *in vitro*. The disparity is probably due to the presence of endogenous ligands for the antibody.

Acknowledgements—We wish to thank Dr Mark R. Bowles for confirming the magnitude of the K_{Ab} *in vitro*. This work was supported by the National Health and Medical Research Council of Australia and P.H.S. Grant MH 42799.

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REFERENCES

1. Pentel PR, Keyler DE, Brunn GJ, Milavetz JM, Altenhofen DG and Pond SM, Redistribution of tricyclic antidepressants in rats using a drug-specific monoclonal antibody: dose–response relationship. *Drug Metab Dispos*, in press.
2. Muller R, Determination of affinity and specificity of anti-hapten antibodies by competitive radioimmunoassay. *Methods Enzymol* **92**: 589–601, 1983.
3. Hogg PJ, Johnston SC, Bowles MR, Pond SM and Winzor DJ, Evaluation of equilibrium constants for antigen–antibody interactions by solid phase immunoassay: the binding of paraquat to its elicited mouse monoclonal antibody. *Mol Immunol* **24**: 797–801, 1987.
4. Duggleby RG, A nonlinear regression program for small computers. *Anal Biochem* **110**: 9–18, 1981.
5. Smith TW, Lloyd BL, Spicer N and Haber E, Immunogenicity and kinetics of distribution and elimination of sheep digoxin-specific IgG and Fab fragments in the rabbit and baboon. *Clin Exp Immunol* **36**: 384–396, 1979.
6. Chauvelot-Moachon L, Delers F, Pous C, Engler R, Tallet F and Giroud JP, Alpha-1-acid glycoprotein concentrations and protein binding of propranolol in Sprague–Dawley and Dark Agouti rat strains treated by phenobarbital. *J Pharmacol Exp Ther* **244**: 1103–1108, 1988.
7. Nyberg G and Martensson E, Determination of free fractions of tricyclic antidepressants. *Naunyn Schmiedebergs Arch Pharmacol* **327**: 260–265, 1984.
8. Scatchard G, The attractions of proteins for small

molecules. *Ann NY Acad Sci* **51**: 660–672, 1949.
9. Pentel PR and Keyler DE, Effects of high dose alpha-1-acid glycoprotein on desipramine toxicity in rats. *J Pharmacol Exp Ther* **218**: 1061–1066, 1988.
10. Pentel PR and Benowitz NL, Efficacy and mechanism of action of sodium bicarbonate in the treatment of desipramine toxicity in rats. *J Pharmacol Exp Ther* **230**: 12–19, 1984.
11. Pentel PR, Keyler DE, Gilbertson DG, Ruth G and Pond SM, Pharmacokinetics and toxicity of high doses of antibody Fab fragments in rats. *Drug Metab Dispos* **16**: 141–145, 1988.

Biochemical Pharmacology, Vol. 41, No. 3, pp. 476–478, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Selective stimulation of carboxylesterases metabolizing charged steroid esters by hydrocortisone

(*Received* 2 *July* 1990; *accepted* 27 *September* 1990)

Corticosteroids have gained wide therapeutic use in the treatment of various diseases. In order to prolong the duration of action or to prepare injectable solutions, many steroid hormones and analogous synthetic derivatives are applied as esters. Studies on the metabolism of these compounds have revealed that the hydrolysis of steroid esters to generate the free molecule is accomplished by a group of steroid esterases of the B-type [1, 2]. We have reported earlier the existence of three distinct carboxylesterases involved in the hydrolysis of steroid esters, where two enzymes are responsible for the metabolism of hydrocortisone hemisuccinate (HCHS) at pH 5.5 and 8.0 and a third enzyme for the metabolism of hydrocortisone acetate (HCAC) at pH 8.0, in isolated rat liver microsomes [3]. The free steroid molecule then undergoes hydroxylation via the cytochrome P450-dependent oxidative pathway and is excreted as its glucuronide conjugate [4].

In a recent report evaluating the responses of mixed function oxidases (MFO) to corticosteroids, cortisone acetate and deoxycortisone acetate elicited substrate specific and sex-dependent changes in arylhydrocarbon hydroxylase and aminopyrene *N*-demethylase activity [5]. In fact, adrenalectomized male rats retrieved the MFO-component enzyme level as well as *N*-demethylase activity upon being administered subcutaneous doses of cortisone acetate (5 mg/kg) for 6 consecutive days [6].

Heterogeneous multiple forms of carboxylesterases are known to be involved in the hydrolytic metabolism of xenobiotics in tissues and blood [7–9]. The hepatic microsomal and cytosolic enzymes belonging to this group [10–12] are nonspecifically induced by phenobarbital and carcinogenic polycyclic aromatic hydrocarbons such as 3,4-benzo(*a*)pyrene and 3-methylcholanthrene. In addition, their interactions with a vast array of xenobiotics renders them susceptible to modulation in a qualitative and quantitative manner [13–15]. Considerable work during the past few years on the influence of hormones on hydrolytic metabolism has failed to spell out a clearly defined role for pituitary, adrenal and gonadal systems [16, 17]. Besides, the influence of repeated administration of corticosteroids on the enzymes hydrolysing xenobiotics remains as yet unassessed.

This prompted us to examine the changes occurring in hepatic microsomal hydrolytic metabolism of hemi-succinate and acetate esters of hydrocortisone, acetyl-salicylic acid (ASA), 2-acetylaminofluorene (AAF), acetanilide, *p*-nitrophenylacetate (NPA), procaine and butyrylcholine, and cytosolic thiacetazone esterase under the effect of repetitive oral treatment with charged and uncharged hydrocortisone esters in rat.

Materials and Methods

Chemicals. 2-Acetylaminofluorene, acetanilide, procaine hydrochloride and bovine serum albumin were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Butyrylcholine iodide and *p*-nitrophenylacetate were acquired from Koch-Light Lab. (Colnbrook, Bucks, U.K.) and Sisco Research Lab. Pvt. Ltd (India), respectively. Hydrocortisone and its hemisuccinate and acetate esters were a gift from Glaxo Laboratories (India) Ltd (Bombay) and thiacetazone from Dey's Medical Stores (Manufacturing) Ltd (India). All other chemicals were of analytical grade.

Male albino rats having free access to food and water were used throughout the study. Rats in batches of six to eight animals each were administered equimolar quantities (≈10 mg/kg HC base) of HC (10 mg/kg), HCAC (13.4 mg/kg) and HCHS (11.2 mg/kg) in 1% gum acacia (w/v) orally with the aid of a feeding cannula for 8 consecutive days. Controls received a comparable quantity of the vehicle. The animals were killed by decapitation 18–20 hr after administration of the last dose. Their livers were removed immediately, chopped, washed in ice-cold 1.15% KCl buffered with 0.01 M Tris–HCl to remove blood and homogenized in the same medium with a Potter–Elvehjem homogenizer. Liver microsomes and cytosol were prepared by differential centrifugation by the procedure described earlier [3].

Assay. Enzyme activity was assayed spectrophotometrically by estimating the quantity of product formed or substrate disappeared. Thus, steroid esterases hydrolysing HCHS at pH 5.5 and 8.0 (I and II) and HCAC at pH 8.0, procaine esterase and butyrylcholine esterase were assayed by following the disappearance of substrates using alkaline hydroxylamine-ferric chloride reagent [18, 19]. The quantity of salicylic acid (at pH 5.5 and 7.4), *p*-nitrophenol, aniline and aminofluorene produced/min/mg protein served as a measure of the activity of microsomal B-esterases hydrolysing the substrates ASA, NPA, acetanilide and AAF [3, 20, 21]. In the cytosol thiacetazone esterase was evaluated on the basis of its hydrolytic product 4-aminobenzaldehyde thiosemicarbazone [22].

Protein was determined by the method of Lowry *et al.* [23] using bovine serum albumin as the reference standard. The Student's *t*-test was employed for the analysis of the results.

Results and Discussion

The activities of hydrolases exemplified in Table 1 reveal a markedly selective augmentation of HCHS esterase activity following oral treatment with HCHS, HCAC and