Biochimica et Biophysica Acta, 659 (1981) 362—369
Elsevier/North-Holland Biomedical Press

BBA 69284

AFFINITY CHROMATOGRAPHY OF HEPATIC GLUTATHIONE *S*-TRANSFERASES ON ω-AMINOALKYL SEPHAROSE DERIVATIVES OF GLUTATHIONE

MASAYASU INOUE *,[a], MICHIAKI HARA [a], FUJIO NAGASHIMA [a], SUNAO MATSUI [a], NOBUO MITSUYASU [b] and YOSHIMASA MORINO [a]

[a] *Department of Biochemistry, Kumamoto University Medical School, 2-2-1, Honjo, Kumamoto 860 and* [b] *Laboratory of Life Chemistry, Faculty of Liberal Arts and Natural Sciencies, Saga University, 1, Honjo-machi, Saga 840 (Japan)*

(Received February 18th, 1981)

Key words: Glutathione S-transferase; Affinity chromatography; ω-Aminoalkyl Sepharose; Glutathione derivative; (Rat liver)

Summary

Rat liver glutathione *S*-transferases (RX: glutathione R-transferase, EC 2.5.1.18) were found to adsorb *S*-carbamidomethyl glutathione linked to Sepharose CL-4B via lysyl or aliphatic diamine spacers of various carbon chain lengths (-NH-$(CH_2)n$-NH-, n = 2, 4, 5, 6, 8 and 10). Proteins were eluted specifically by reduced glutathione. The affinity of the enzymes for the adsorbent increased with increase in the carbon chain length of aliphatic diamine spacers used. Adsorbent having a free carboxyl group within the spacer moiety had high capacity and was specific for glutathione *S*-transferases. The transferases were specifically eluted from the column in high yield by low concentrations of glutathione. Enzymes purified by the lysyl spacer adsorbent were homogeneous in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and contained most of the hepatic glutathione *S*-transferase isozymes in isoelectric focusing. Oxidized glutathione and *S*-methyl glutathione were equally effective as reduced glutathione in eluting glutathione *S*-transferases from the adsorbent, but γ-glutamylcysteinylglycineamide or γ-glutamylcysteinylglycine-1-methyl ester were not effective. These data suggested that the free carboxyl group of glycyl moiety of glutathione might also be important for the specific binding of the transferases to this adsorbent.

* Present address: Liver Research Center, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, U.S.A.

0005-2744/81/0000—0000/$02.50 © Elsevier/North-Holland Biomedical Press

Introduction

Glutathione *S*-transferases (RX: glutathione R-transferase, EC 2.5.1.18) catalyze the conjugation of glutathione with a wide variety of electrophiles and are believed to be involved in the non-oxidative detoxication of xenobiotics [1—4]. This reaction is thought to be an initial step of mercapturic acid biosynthesis. Most of xenobiotics that can serve as substrates for these transferases contain hydrophobic substituents. Glutathione *S*-transferase B, a major component of the transferases, is identical to ligandin, which is also a hepatic binding protein for organic anion such as bilirubin [5,6]. Like ligandin, the other transferases also show some binding capacity for these organic anions. Based on these findings, glutathione *S*-transferases have been thought to be one of the major determinants for the net flux of organic anions from plasma into the liver [7]. Thus, these transferases appear to possess at least two distinct sites, i.e., the site for glutathione and that for hydrophobic anions. Knowledge of the topographical correlation between these two binding sites may be important for understanding the mechanism of the enzyme catalysis as well as its physiological function as the organic anion transporters. Affinity chromatography can be utilized not only as a tool for the enzyme purification [8—11], but also for the study of the enzyme-ligand interactions [12]. The present communication describes an attempt to characterize the ligand binding sites in the glutathione *S*-transferases by using affinity chromatography techniques.

Materials and Methods

Materials. Sepharose CL-4B was obtained from Pharmacia Chemical Co. Chloroacetyl chloride, 2,4-dinitrochlorobenzene and aliphatic diamines of various chain lengths (NH_2-(CH_2)-*n*-NH_2; n = 2, 4, 5, 6, 8 and 10) were purchased from Nakarai Chemical Co. (Kyoto). Reduced glutathione and *S*-methyl glutathione were obtained from Sigma Chemical Co. Other reagents used were of analytical grade.

Synthesis of glutathione-conjugated Sepharose CL-4B. ω-Aminoalkyl Sepharose derivatives were synthesized by coupling lysine or aliphatic diamines of different carbon chain length to Sepharose CL-4B (0.25 mmol/g wet wt. gel) by the method of Axen and Ernback [8]. To ω-aminoalkyl Sepharose suspended in 20% dioxan, chloroacetyl chloride (0.75 mmol/g wet wt. ω-aminoalkyl Sepharose) was added and the mixture was kept at pH 7.0—8.0 by adding 2 N KOH at 4°C for 30 min. The resulting gel was successively washed with 100 vol. each of H_2O, 1 M NaCl/H_2O. The chloroacetyl derivative of an ω-aminoalkyl Sepharose was incubated with reduced glutathione (0.15 mmol/g wet wt. chloroacetyl Sepharose) dissolved in 0.2 M Tris-HCl buffer, pH 8.0 for 12 h under nitrogen stream. The resulting *S*-carbamidomethyl glutathione derivative of an ω-aminoalkyl Sepharose was further incubated with 2-mercaptoethanol (1 mmol/g wet wt. gel), for 24 h at 37°C, to eliminate unreacted chloroacetyl group, and washed successively with 100 vol. each of H_2O, 1 M NaCl, 0.1 M $NaHCO_3$/H_2O.

Enzyme samples. A partially purified preparation of glutathione *S*-transferase was obtained from frozen rat liver of male Wistar rats by the method

of Habig et al. [13]. Rat livers (100 g) were homogenized with 300 ml water in a Waring Blendor for 2 min and the homogenate was centrifuged at 10 000 × *g* for 60 min. The supernatant fluid was passed through a column of DEAE-cellulose (Whatman DE-52, 6 × 20 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The column was rinsed with 500 ml buffer solution as described above and unadsorbed fractions were collected. The $(NH_4)_2SO_4$ precipitated fraction (35—70%) was obtained from the combined solution as described [13]. After dialysis for 20 h at 4°C against 5 l of 10 mM potassium phosphate buffer, pH 7.0, this crude enzyme preparation was used for affinity chromatography (1.4 unit/mg protein).

Assay for enzyme activity. Glutathione *S*-transferase activity was measured at 25°C by using 2,4-dinitrochlorobenzene as described by Habig et al. [13]. Protein concentration was determined by the method of Lowry et al. [14] using bovine serum albumin as a standard.

Affinity chromatography. Columns containing 3 ml of various *S*-carbamidomethyl glutathione affinity derivatives of Sepharose were equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The enzyme samples (1—2 ml) were passed over the column, followed by washing with the same buffer solution. Elution of the enzyme from the column was carried out with 10 mM Tris-HCl buffer, pH 8.0/5 mM reduced glutathione at a flow rate of 10 ml/h, unless otherwise indicated. Fractions of 2 ml were collected and assayed for enzyme activity.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed at pH 7.2 as described previously [15] by the method of Weber and Osborn [16]. After electrophoresis, protein was visualized by staining with Coomassie brilliant blue R-250. Densitometric scanning of the stained gels was carried out in a Shimazu Dual-Wavelength Scanner CS-900 at 550 nm for the protein staining.

Isoelectric focusing. The enzyme sample (200 units) obtained by affinity chromatography was subjected to isoelectric focusing (pH range of 3.0—10.0) as described [17].

Results

Importance of hydrophobic character of the spacer moiety

Fig. 1 compares the chromatographic patterns of glutathione *S*-transferases on the ω-aminoalkyl spacer adsorbents. The derivatized Sepharose having ethylenediamine as the spacer (Fig. 1a) excluded about one-half of the enzyme activity. The enzyme activity adsorbed was eluted from the column with reduced glutathione. The increase in the carbon chain length of the aminoalkyl spacers resulted in the increase of the capacity to adsorb the enzyme. The enzyme activity was fully adsorbed to the derivatized Sepharose having octanediamine or decamethylenediamine as the spacer (Fig. 1d and e). In all cases, glutathione was equally effective as an elutant of the transferases from the adsorbents. However, the enzyme activity was not eluted with the same concentration of KCl or sodium glutamate as that of glutathione (data not shown).

Importance of a carboxyl group in the spacer moiety

It is well known that glutathione *S*-transferases preferentially bind nega-

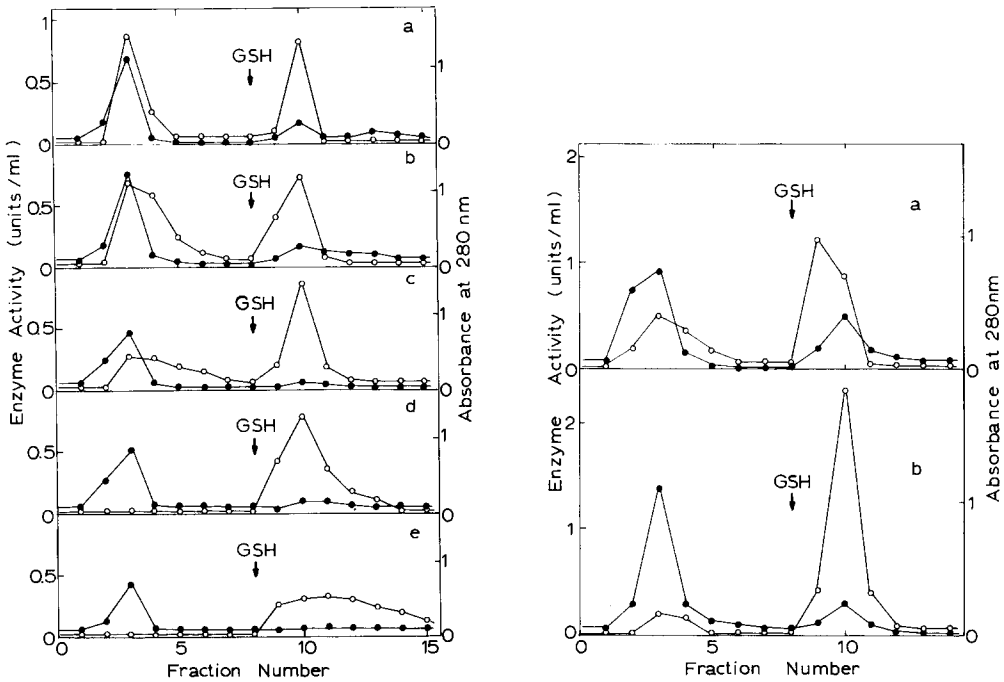

Fig. 1. Affinity chromatography of gluthatione *S*-transferases on glutathione-conjugated Sepharose columns containing various spacers. The enzyme samples containing 5 units glutathione *S*-transferase activity were applied to the column (1 × 3.8 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. After washing with 16 ml buffer solution, elution of the enzyme was performed with 10 mM Tris-HCl buffer, pH 8.0/5 mM reduced glutathione (2 ml/fraction). All procedures were carried out at 4°C. Spacers used were: a, ethylenediamine; b, propanediamine; c, hexanediamine; d, octanediamine; e, decamethylenediamine. Arrow shows the change in the elution buffer solution. GSH, reduced glutathione. ○———○, enzyme activity; ●———●, absorbance at 280 nm.

Fig. 2. Chromatography on the lysyl or pentanediamine spacer adsorbent. The enzyme samples (7 units) were applied to the columns of lysyl or pentanediamine spacer adsorbent (1 × 3.8 cm). Elution of the enzyme was carried out with 5 mM glutathione. Other conditions were the same as in Fig. 1. a, pentanediamine spacer adsorbent; b, lysyl spacer adsorbent. ○———○, enzyme activity; ●———●, absorbance at 280 nm.

tively-charged hydrophobic compounds. Thus, it seems reasonable that the introduction of a negatively charged group into hydrophobic spacer moiety of the derivatized Sepharose might increase the affinity for the transferases. For this purpose, *S*-carbamidomethyl glutathione derivative of Sepharose having a lysyl spacer was synthesized. This derivative should possess an additional free carboxyl group on the spacer moiety structurally corresponding to diaminopentane. Fig. 2 compares the chromatographic pattern of the transferases on the *S*-carbamidomethyl glutathione derivative of diaminopentyl- or lysyl Sepharose. The lysyl spacer adsorbent seemed to show higher affinity for the transferases than the diaminopentane spacer adsorbent, although they are identical in carbon chain length. In addition, it should be noted that, in contrast to the case of the adsorbent prepared by using other spacers, the enzyme adsorbed to the lysyl spacer adsorbent was eluted with reduced glutathione as a particular sharp peak. Purification in this single step was about 13-fold and the recovery of the transferases was higher than 95%.

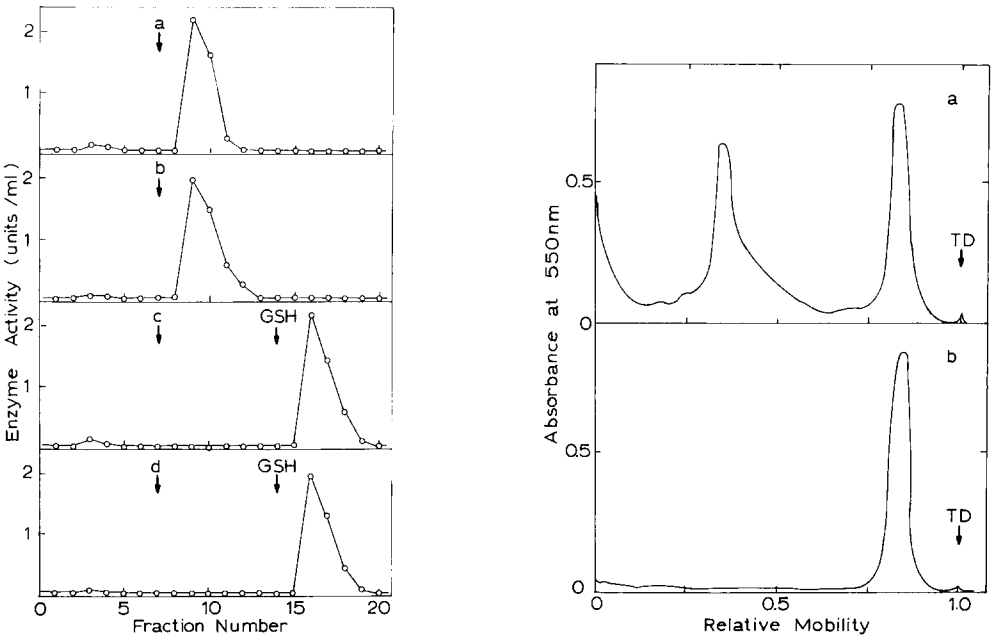

Fig. 3. Elution of the enzymes by various glutathione derivatives. The enzyme samples (10 units) were applied on a column of the lysyl spacer adsorbent (1 X 3.8 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. After washing with 14 ml of the same buffer solution as described above, elution was carried out with 10 mM Tris-HCl buffer, pH 8.0, containing 5 mM of various glutathione derivatives. Other conditions were the same as in Fig. 1. Elution by (a) oxidized glutathione, (b) *S*-methyl glutathione, (c) γ-glutamylcysteinylglycineamide and (d) γ-glutamylcysteinylglycine-1-methyl ester. GSH, reduced glutathione.

Fig. 4. Polyacrylamide gel electrophoresis of the enzyme. The enzyme samples (50 μg) purified by using the column of (a) the diaminopentyl or (b) the lysyl spacer adsorbent were denatured by incubating with 1% SDS and 2-mercaptoethanol at 100°C for 3 min. Electrophoresis was carried out with 7% polyacrylamide gels in the presence of 0.1% SDS. TD, tracking dye.

Importance of a carboxyl group in the glycyl moiety of glutathione

As shown in Fig. 3, reduced glutathione as an elutant was replaced by oxidized glutathione or *S*-methyl glutathione. However, γ-glutamylcysteinylglycineamide and γ-glutamylcysteinylglycine-1-methyl ester were found to be ineffective in eluting the enzyme from the column. Preliminary experiments showed that the latter two glutathione derivatives were poor substrates for glutathione *S*-transferases (data not shown).

Purification of glutathione S-transferase isozymes

The enzyme preparation specifically eluted from the lysyl spacer adsorbent with reduced glutathione showed much higher specific activity than those obtained by chromatography on other adsorbents. Upon SDS-polyacrylamide gel electrophoresis, this enzyme sample revealed a single protein band (Fig. 4). Molecular weight of this band, estimated by SDS-polyacrylamide gel electrophoresis on the basis of standardization with proteins of known molecular weight (as in Ref. 15) was 23 000, which was identical to that of the transferase subunits [7]. Rather marked contaminant protein bands were also observed

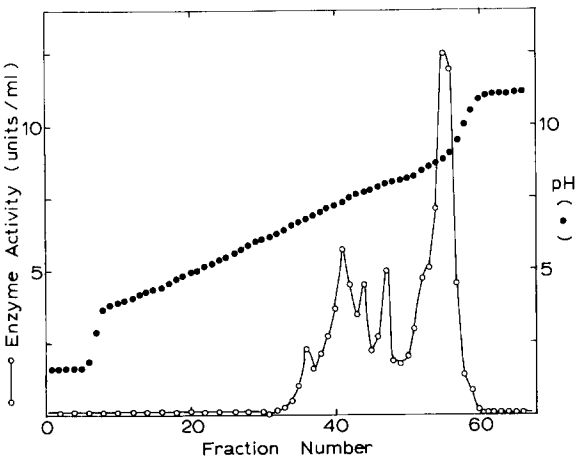

Fig. 5. Isoelectric focusing of the purified enzymes. Glutathione *S*-transferase preparations purified by affinity chromatography on the lysyl spacer adsorbent were subjected to isoelectric focusing. After isoelectric focusing, fractions of 2 ml were collected and determined for the enzyme activity (○———○) and pH (●———●).

with enzyme preparations purified by using other adsorbents (data not shown). Isoelectric focusing of the enzyme sample purified by the lysyl spacer adsorbent revealed six fractions of glutathione *S*-transferase activity having different p*I* values (Fig. 5), suggesting that the purified enzyme preparation contained most of the transferase isozymes.

Discussion

The affinity of rat liver glutathione *S*-transferases for an *S*-carbamidomethyl glutathione derivative of an ω-aminoalkyl Sepharose was found to increase with increasing carbon chain length of aliphatic diamine spacers employed. These findings suggest that hydrophobic character of the spacer moiety might be important for the interaction of the adsorbent with the enzymes. Among various spacers tested, the lysyl spacer was found to be the most specific for glutathione *S*-transferase. This might be due to the fact that, besides the glutathione binding site, the transferases possess another binding site responsible for the interaction with hydrophobic anions [18]. The full scope and details of the mechanism of interaction of glutathione *S*-transferases with ligands are yet to be realized, but there are a number of potential implications that may be derived from these data. For example, it would be important to know the mode of linkage between the spacer lysyl moiety and ligand glutathione for understanding the topographical relationship between the two different binding subsites in the transferases. Since the p*K* value of α-amino group of lysine is much lower than that of ϵ-amino group, spacer lysine might bind to the activated gel mainly via its α-amino group under the present coupling conditions (pH 8.0). Thus, the free carboxyl group of spacer lysine might be located distal to the glutathione moiety. It would, therefore, be interesting to compare the affinity chromatographic properties of the transferases on this adsorbent with that of an adsorbent in which the lysyl spacer bound to Seph-

arose via its ϵ-amino group, since the free α-carboxyl group of lysyl moiety might be located proximal to the glutathione moiety. As judged from the studies on elution of the enzyme adsorbed to the lysyl spacer adsorbent (Fig. 3), the free thiol group of glutathione does not seem to be necessary for its binding to the enzyme. On the other hand, the carboxyl group of the glycyl moiety is probably essential since the transferases were eluted from this column either by oxidized glutathione or *S*-methyl glutathione but not by glutathione derivatives the carboxyl group of which were blocked in the glycyl moiety (amide or methyl ester). This is consistent with the fact that both oxidized glutathione and *S*-methyl glutathione could serve as inhibitors of the transferase, while the latter two derivatives are rather poor substrates for the enzyme (unpublished data).

Apart from information about the intrinsic mechanism of binding of the transferases to the adsorbent, the lysyl spacer adsorbent is expected to provide an useful tool for a rapid and extensive purification of the enzyme. In fact, the adsorbent prepared in the present study was capable of binding approx. 100 units of glutathione *S*-transferase/ml gel. The purification factor for the crude enzyme sample was about 13-fold. This adsorbent was also found to be effective for unfractionated liver cytosol (about 18-fold enrichment in a single chromatographic step). The purified enzyme sample by this single step of chromatography showed a major protein band corresponding to that of the subunits of the transferases with slight contaminant bands upon SDS-polyacrylamide gel electrophoresis (data not shown).

Sulfobromophthalein has been used for the affinity chromatography of glutathione *S*-transferases [9—11]. However, this ligand binds tightly to the transferases, thus causing difficulties in the subsequent elution of the enzymes from the adsorbent with good recovery [19]. Furthermore, this dye showed rather broad binding to a variety of cellular constituents especially at less than neutral pH values [20—21], presumably due to its strong negative charge. The major advantages of the lysyl spacer adsorbent might be that the spacer moiety provided a moderate hydrophobicity and negative charge necessary for specific, reversible interaction of the adsorbent with glutathione *S*-transferases.

Acknowledgements

We wish to express our gratitute to Dr. I. Listowsky for the stimulating discussions which contributed to the final form of the manuscript.

References

1 Booth, J. and Boyland, E. (1961) Biochem. J. 79, 510—524
2 Boyland, E. and Chasseaud, L.F. (1960) Adv. Enzymol. 32, 173—219
3 Wood, W.L. (1970) in Metabolic Conjugation and Metabolic Hydrolysis (Fishman, W.H., ed.), Vol 2, pp. 261—299, Academic Press, New York
4 Jakoby, W.B. (1978) in Function of Glutathione in the Liver and Kidney (Sies, H and Wendel, A., ed.), pp. 157—163, Springer-Verlag, New York
5 Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M. and Jakoby, W.B. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3879—3882
6 Kirsch, R., Fleischner, G., Kamisaka, K. and Arias, I.M. (1975) J. Clin. Invest. 55, 1009—1019
7 Arias, I.M., Fleischner, G., Kirsch, R., Mishkin, S. and Gatmaitan, Z. (1975) in Glutathione: Metabolism and Function (Arias, I.M. and Jacoby, W.B., eds.), pp. 175—188, Raven Press, New York

8 Axén, R. and Ernback, S. (1971) Eur. J. Biochem. 18, 351—360
9 Grahnén, A. and Sjöholm, I. (1977) Eur. J. Biochem. 80, 573—580
10 Clark, A.G., Letoa, M. and Ting, W.S. (1977) Life Sci. 20, 141—148
11 Wolkoff, A.W., Bhargava, M.M., Chung, C. and Gatmaitan, Z. (1979) Proc. Soc. Exptl. Biol. Med. 160, 150—153
12 Lowe, C.R. and Dean, P.D.G. (1974) in Affinity Chromatography pp. 12—89, John Wiley and Sons, New York
13 Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) J. Biol. Chem. 249, 7130—7139
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Inoue, M., Horiuchi, S. and Morino, Y. (1977) Eur. J. Biochem. 73, 335—342
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
17 Horiuchi, S., Inoue, M. and Morino, Y. (1978) Eur. J. Biochem. 87, 429—437
18 Bhargava, M.M., Listowsky, I. and Arias, I.M. (1978) J. Biol. Chem. 253, 4112—4115
19 Guthenberg, C. and Mannervik, B. (1979) Biochem. Biophys. Res. Commun. 86, 1304—1310
20 Killenberg, P.G. and Hoppel, C.L. (1974) Mol. Pharmacol. 10, 108—118
21 Burr, R., Schwenk, M. and Pfaff, E. (1977) Biochem. Pharmacol. 26, 461—466
22 Wolkoff, A.W. and Chung, C.T. (1980) J. Clin. Invest. 65, 1152—1161