Biochem. J. (1980) **188**, 889–893
Printed in Great Britain

The Effect of Vitamin A Deficiency on Hepatic, Renal and Pulmonary Glutathione *S*-Transferase Activities in the Rat

Zahid H. SIDDIK,* Edward G. MIMNAUGH, Michael A. TRUSH and Theodore E. GRAM†
Laboratory of Toxicology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205, U.S.A.

(*Received 8 October 1979*)

Feeding male weanling rats on a vitamin A-deficient diet for 6 weeks resulted in significant increases (44–57%) in glutathione *S*-aryl-, *S*-aralkyl- *S*-alkyl- and *S*-epoxidetransferase activities in the liver cytosol. Only the *S*-aralkyl- (27%) and *S*-alkyltransferase (14%) activities were signficantly increased in the kidney as a result of deficiency. There was no effect on any of the pulmonary glutathione *S*-transferase activities. The increases in hepatic transferase activities were due primarily to increases (25–96%) in the apparent $V_{max.}$. There were no changes in the apparent K_m of any of the four drug substrates employed. With 3,4-dichloronitrobenzene as the second substrate, the apparent K_m for glutathione was increased by over 2-fold in vitamin A-deficient livers as compared with controls. The relationship between these results and enhanced susceptibility to chemical carcinogens in vitamin A deficiency is briefly discussed, and comparison is made between the effects of this nutritional state and pretreatment with drug inducers on the glutathione *S*-transferases.

Glutathione *S*-transferases (EC 2.5.1.18) are important cytosolic enzymes that catalyse the metabolism of diverse foreign compounds, including chemical carcinogens (Chasseaud, 1976). It is also known that these transferases function non-enzymically to bind certain drugs both reversibly and covalently and thereby decrease their potential toxicity (Smith *et al.*, 1977). An inverse correlation between glututhione *S*-transferase activity and susceptibility to chemical carcinogenesis has been proposed (Smith *et al.*, 1977), and the prevention of the neoplastic effects of chemicals by butylated hydroxyanisole and ethoxyquin have been partly attributed to large increases in hepatic glutathione *S*-transferase activities after dietary intake of these antioxidants (Benson *et al.*, 1978).

Enhanced susceptibility to carcinogens in vitamin A deficiency and, conversely, prevention of chemical carcinogenesis by vitamin A and its analogues (retinoids) have been the subject of numerous studies (see review by Sporn *et al.*, 1976). The increase in the incidence of tumours in the respiratory tract of vitamin A-deficient animals in response to benzo[*a*]pyrene correlates with increased binding of the carcinogen to DNA after metabolic activation (Genta *et al.*, 1974). Enhanced tissue binding in deficiency may arise if a greater concentration of the free carcinogenic metabolite is achieved intracellularly, possibly through an impaired catabolism of the active metabolite by glutathione *S*-transferases. In the present investigation we have therefore examined the effect of vitamin A deficiency on hepatic, renal and pulmonary glutathione *S*-aryl-, *S*-aralkyl-, *S*-alkyl- and *S*-epoxidetransferase activities.

This work was presented in part at the Fourth International Symposium on Microsomes and Drug Oxidations, Ann Arbor, MI, U.S.A., July 15–18, 1979 (Siddik *et al.*, 1979).

Abbreviations used: GSH, reduced glutathione; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

* Present address: Department of Biochemical Pharmacology, Institute of Cancer Research: Royal Cancer Hospital, Belmont, Sutton, Surrey SM2 5PX, U.K.

† To whom reprint requests should be addressed.

Materials and Methods

Chemicals

1,2-Dichloronitrobenzene and 4-nitrobenzyl chloride were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. 1,2-Epoxy-3-(4-nitrophenoxy)propane and [^{14}C]methyl iodide (specific radioactivity 59.7Ci/mol) were purchased from Eastman Kodak Co., Rochester, NY, U.S.A. and Amersham Corp., Arlington Heights, IL, U.S.A. respectively. All other compounds were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Animals

Male weanling Sprague-Dawley rats (23 days old;

0306-3283/80/060889-05$01.50/1

60–65g) were obtained from Taconic Farms, Germantown, NY, U.S.A. and maintained for 6 weeks on either a vitamin A-deficient diet (less than 20i.u./kg; ICN Pharmaceuticals, Cleveland, OH, U.S.A., catalogue no. 104646) or an identical diet supplemented with retinyl acetate (20000i.u./kg). Animals were allowed food and water *ad libitum* during the first 4 weeks. Thereafter, when the anorexic effect of vitamin A deficiency became pronounced, the amount of diet supplied to the control animals was reduced in proportion to that consumed by the group on the deficient diet.

Preparation of cytosol

The rats were killed by a blow to the head, and livers, lungs and kidneys were quickly excised and rinsed in cold KCl/Tris buffer (150mM-KCl/50mM-Tris/HCl), pH 7.4. Subsequent procedures were conducted at 0–4°C. The tissues were minced with scissors and homogenized in 2vol. of KCl/Tris buffer using a motor-driven Teflon-and-glass tissue grinder (size C; A. H. Thomas and Co., Philadelphia, PA, U.S.A.). The resulting homogenates were diluted to 25% (w/v) with KCl/Tris buffer and centrifuged at 105000*g* for 60min in a Beckman L3-50 ultracentrifuge (Beckman Instruments, Fullerton, CA, U.S.A.). The cytosols (supernatant fractions) were carefully removed, avoiding the floating lipid pellicles, and diluted to 6mg of protein/ml with KCl/Tris buffer.

Analytical procedures

Enzyme assays were conducted under conditions giving activities that were linear with respect to protein concentrations and incubation times.

Glutathione *S*-aryl-, *S*-aralkyl- and *S*-epoxide-transferase activities were determined at 37°C by measuring product formation spectrophotometrically at 350, 310 and 360nm respectively as described previously (Grover & Sims, 1964; Fjellstedt *et al.*, 1973; Habig *et al.*, 1974). Incubations contained 10mM-GSH, 50–500μl of diluted cytosol and the appropriate buffer in a final volume of 2.85ml. The *S*-aryltransferase activity was determined in 0.4M-Hepes, pH 8.3, and the *S*-aralkyl- and *S*-epoxidetransferase activities were determined in 0.1M-potassium phosphate, pH 6.5 and 7.4, respectively. Reactions were initiated by the addition of 3,4-dichloronitrobenzene (a substrate for glutathione *S*-aryltransferase; 1.0mM final concentration), 4-nitrobenzyl chloride (*S*-aralkyltransferase; 1.0mM) and 1,2-epoxy-3-(4-nitrophenoxy)-propane (*S*-epoxidetransferase; 0.5mM) in ethanol (100μl). All enzyme activities were corrected for non-enzymic interaction between substrates.

Glutathione *S*-alkyltransferase activity was determined by the procedure of Johnson (1966) as described by Kaplowitz *et al.* (1975). Reactions were conducted at 25°C in 1.0ml of 66mM-sodium phosphate/2mM-EDTA buffer, pH 7.0, containing 10mM-GSH and diluted cytosol (100–200μl), and were initiated by the addition of [^{14}C]methyl iodide (5.0mM final concentration; sp. radioactivity 60μCi/mmol) in ethanol (50μl). Samples (100μl) were removed at 0 (blank value) and 5min, added to an equal volume of methanol in a liquid-scintillation vial to stop the reaction and the mixture was evaporated to dryness. Hot distilled water (1.0ml) was then added, followed by 18ml of ACS scintillant (Amersham Corp.), and the radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer (model 3390) at a counting efficiency of 66–68%.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Tissue vitamin A concentrations, expressed as retinol equivalents, were measured as described by Dugan *et al.* (1964).

Statistical analyses were performed by using Student's *t* test for significance at the $P < 0.05$ level.

Enzyme kinetics

The hepatic glutathione *S*-transferase activities were determined using 10mM-GSH and the following range of substrate concentrations: 3,4-dichloronitrobenzene, 0.1–1.0mM; 4-nitrobenzyl chloride, 0.1–1.0mM; [^{14}C]methyl iodide (specific radioactivity 60–200μCi/mmol), 0.5–5.0mM; 1,2-epoxy-3-(4-nitrophenoxy)propane, 0.1–0.5mM. The kinetic parameters for GSH (0.1–1.0mM) were determined with 3,4-dichloronitrobenzene (1.0mM) as the second substrate. Apparent K_m and $V_{max.}$ values were calculated by finding the best fit of the data to the Michaelis–Menten hyperbolic equation by using a reiterative least-squares computer program, MLAB, developed by the Division of Computer Research and Technology, National Institutes of Health (Knott & Reese, 1972).

Results

Body and organ weights, and vitamin A and protein concentrations

There were no differences between control and deficient animals in the growth rate, body and organ weights and cytosol protein concentrations of liver, lung and kidney after feeding rats on the appropriate diet for 6 weeks (Table 1). Vitamin A concentrations in the three organs, on the other hand, were significantly reduced in animals fed on the deficient diet, being less than 1μg/g compared with about 5, 7 and 207μg/g in control kidney, lung and liver respectively (Table 1).

Enzyme activities in liver, lung and kidney

Glutathione *S*-transferase activities are presented in Table 2. Vitamin A deficiency resulted in

Table 1. *Status of rats after receiving a vitamin A-deficient diet for 6 weeks*

Male weanling rats (60–65 g) were maintained for 6 weeks either on a vitamin A-deficient diet or on an identical diet supplemented with retinyl acetate (20000 i.u./kg). Results are expressed as means ± S.D. of the number of determinations shown in parentheses. N.S., no significant difference between control and deficient groups ($P > 0.05$). See the text for experimental details.

	Control	Deficient	
Mean daily growth rate (g/day):			
0–4 weeks	5.4 ± 0.6 (15)	5.2 ± 0.6 (15)	N.S.
5–6 weeks	3.9 ± 0.5 (15)	3.5 ± 0.4 (15)	N.S.
Body weight (g)	266 ± 24 (15)	258 ± 19 (15)	N.S.
Organ weights (g):			
Liver	13.6 ± 2.1 (15)	12.7 ± 1.9 (15)	N.S.
Lung	1.73 ± 0.33 (10)	1.59 ± 0.25 (10)	N.S.
Kidney	1.85 ± 0.26 (10)	1.89 ± 0.23 (10)	N.S.
Vitamin A content (μg/g):			
Liver	207 ± 34 (10)	0.85 ± 0.81 (10)	$P < 0.001$
Lung	6.8 ± 2.3 (5)	0.79 ± 0.25 (5)	$P < 0.001$
Kidney	4.9 ± 0.2 (5)	0.80 ± 0.25 (5)	$P < 0.001$
Cytosol protein (mg/g):			
Liver	56.0 ± 6.3 (10)	51.7 ± 6.7 (10)	N.S.
Lung	65.6 ± 7.5 (5)	63.4 ± 5.1 (5)	N.S.
Kidney	49.4 ± 5.0 (5)	44.5 ± 3.6 (5)	N.S.

Table 2. *Effect of vitamin A deficiency on hepatic, pulmonary and renal glutathione S-transferase activities*

Male weanling rats (60–65 g), maintained for 6 weeks on either a vitamin A-deficient diet or an identical diet supplemented with retinyl acetate (20000 i.u./kg), were killed, and the livers, lungs and kidneys were used to prepare the cytosols as source of the enzymes. The glutathione S-aryl-, S-aralkyl-, S-alkyl- and S-epoxidetransferase activities were determined with 3,4-dichloronitrobenzene, 4-nitrobenzyl chloride, methyl iodide and 1,2-epoxy-3-(4-nitrophenoxy)propane as the respective substrates. Enzymes activities (nmol/min per mg of protein) are expressed as the means ± S.D. of five determinations. Results of statistical comparisons between control and deficient groups are given in parentheses. N.S., no significant difference between control and deficient groups ($P > 0.05$). See the text for experimental details.

	Glutathione S-transferase activities					
	Liver		Lung		Kidney	
Glutathione S-transferase	Control	Deficient	Control	Deficient	Control	Deficient
Aryl	77.4 ± 13.9	118 ± 16 ($P < 0.01$)	1.8 ± 0.4	1.6 ± 0.3 (N.S.)	3.9 ± 1.1	3.9 ± 0.4 (N.S.)
Aralkyl	459 ± 85	719 ± 65 ($P < 0.001$)	42.2 ± 8.3	35.5 ± 5.2 (N.S.)	243 ± 50	308 ± 20 ($P < 0.05$)
Alkyl	82.6 ± 10.5	119 ± 10 ($P < 0.001$)	15.0 ± 2.8	19.2 ± 2.8 (N.S.)	62.0 ± 3.8	70.6 ± 3.8 ($P < 0.02$)
Epoxide	125 ± 11	190 ± 12 ($P < 0.001$)	12.9 ± 2.8	11.6 ± 2.4 (N.S.)	118 ± 26	137 ± 42 (N.S.)

significant increases in glutathione S-aryl- (53%) S-aralkyl- (57%), S-alkyl- (44%) and S-epoxide-transferase (52%) activities in the liver. In the kidney, S-aralkyl- (27%) and S-alkyltransferase (14%) activities were also significantly increased in deficient animals, although the increases were not as great as in the liver. The activities of the renal S-aryl- and S-epoxidetransferases were unchanged. Vitamin A deficiency had no effect on any of the four glutathione S-transferase activities in the lung.

Addition *in vitro* of retinol or retinyl palmitate (equivalent to 400–800 μg/g of liver) to the liver cytosol from rats fed on the deficient diet had no effect on enzyme activity.

Apparent enzyme kinetic parameters

K_m and $V_{max.}$ values were determined to evaluate the effects of deficiency on the kinetics of the four hepatic enzyme conjugation reactions. As shown in Table 3, vitamin A deficiency significantly increased

Table 3. *Effect of vitamin A deficiency on apparent kinetic parameters for hepatic glutathione S-transferase activity*
Male weanling rats (60–65 g), maintained for 6 weeks on either a vitamin A-deficient diet or an identical diet supplemented with retinyl acetate (20000 i.u./kg), were killed and the livers were used as a source of the cytosol enzymes. The glutathione *S*-transferase corresponding to the appropriate substrate is given in the legend to Table 2. The kinetic parameters for GSH were obtained with 3,4-dichloronitrobenzene as the second substrate. The kinetic parameters are expressed as the means ± S.D. of five determinations. Results of statistical comparison between control and deficient groups are given in parentheses. N.S., no significant difference between control and deficient groups ($P > 0.05$). See the text for experimental details.

	Apparent kinetic parameters			
	K_m (mM)		$V_{max.}$ (nmol/min per mg of protein)	
Substrate	Control	Deficient	Control	Deficient
3,4-Dichloronitrobenzene	2.9 ± 0.7	3.6 ± 0.5 (N.S.)	297 ± 46	536 ± 89 ($P < 0.001$)
4-Nitrobenzyl chloride	0.40 ± 0.06	0.43 ± 0.10 (N.S.)	636 ± 125	1012 ± 154 ($P < 0.01$)
Methyl iodide	1.4 ± 0.1	1.5 ± 0.3 (N.S.)	129 ± 9	161 ± 21 ($P < 0.05$)
1,2-Epoxy-3-(4-nitrophenoxy)propane	0.66 ± 0.21	0.78 ± 0.18 (N.S.)	301 ± 59	466 ± 88 ($P < 0.01$)
GSH	0.18 ± 0.05	0.39 ± 0.11 ($P < 0.01$)	56.6 ± 6.6	111 ± 25 ($P < 0.002$)

the maximal enzymic rate ($V_{max.}$) of conjugation of GSH (saturating concentration) with 3,4-dichloronitrobenzene (81%), 4-nitrobenzyl chloride (59%), methyl iodide (25%) and 1,2-epoxy-3-(4-nitrophenoxy)propane (55%). The apparent $V_{max.}$ for conjugation between GSH and 3,4-dichloronitrobenzene, as determined by varying the GSH concentration, was increased by 96% in animals fed on the deficient diet. The apparent K_m for GSH was similarly increased (117%) in the livers from rats fed on the deficient diet (Table 3). However, the apparent K_m values for the other four substrates were unchanged in vitamin A deficiency.

Discussion

Drug metabolism in vitamin A deficiency has been studied by several groups. These investigators, concentrating mainly on the microsomal cytochrome *P*-450-dependent (phase 1) reactions, have reported that activities of several enzymes are decreased in deficiency (Yonemoto & Oh, 1969; Becking, 1973; Colby *et al.*, 1975; Chhabra *et al.*, 1977). Conjugation (phase 2) reactions, on the other hand, have been largely ignored in vitamin A deficiency. However, a brief report by Chhabra *et al.* (1977) indicated that the hepatic and pulmonary glutathione *S*-transferase activities were not affected in vitamin A-deficient rabbits and guinea pigs. This is partly in contrast with the present findings that indicate that several hepatic and renal glutathione *S*-transferase activities were significantly increased in deficient rats. This, however, may be due to a species difference in the response to vitamin A deficiency.

The increases in hepatic glutathione *S*-transferase activities in vitamin A deficiency resemble qualitatively those seen after induction with phenobarbital, 3,4-benzopyrene or 3-methylcholanthrene (Kaplowitz *et al.*, 1975). In the rat kidney, however, these three agents induced the glutathione *S*-aryl- and *S*-aralkyltransferase activities, but had no effect on the *S*-alkyl- and *S*-epoxidetransferase activities (Clifton *et al.*, 1975), whereas in vitamin A deficiency only the *S*-aralkyl- and *S*-alkyltransferase activities were significantly increased. The glutathione *S*-transferase activities in the lung, on the other hand, were increased by pretreatment with phenobarbital (Smith *et al.*, 1978), but not by other inducing agents (Moron *et al.*, 1979; Smith *et al.*, 1978) or by vitamin A deficiency. Preliminary results indicate that extension of the deficiency for up to 10 weeks still had no effect on the renal and pulmonary glutathione *S*-aryltransferase activity.

The increases in $V_{max.}$ of the hepatic glutathione *S*-transferases in vitamin A deficiency indicate that the amounts of these enzymes had increased by presently unknown mechanisms. The increases in the transferase enzymes appear to be a specific response to deficiency, since the total hepatic cytosol protein concentration was unchanged. Although the K_m values of the four xenobiotics were not altered, that of GSH increased over 2-fold in deficiency, indicating an alteration in affinity of the enzyme for this endogenous substrate. This increase in K_m may not be of any great significance in the metabolism of

most drugs *in vivo*, since, in vitamin A-deficient rats, the hepatic intracellular GSH concentration of over 5mM (Z. H Siddik, R. Drew & T. E. Gram, unpublished work), which is more than 10-fold greater than the K_m for GSH in most conjugation reactions (Habig *et al.*, 1974; Baars *et al.*, 1978) (Table 3), would be sufficient to saturate the enzymes with GSH, and thus not affect the rate of metabolism of the xenobiotics. Similarly, the quantitative increases in the activities of the glutathione *S*-transferases seen in vitamin A deficiency were probably not affected by the increase in K_m for GSH, owing to the high concentration of GSH (10mM) employed in the enzyme assays carried out *in vitro*.

The present study therefore indicates that vitamin deficiency increases glutathione *S*-transferase activities in rat liver and kidney, but not lung. The increase in liver contrasts with the general depression of hepatic mono-oxygenase activities in this condition. Five glutathione *S*-transferases exist, possessing overlapping specificities (Jakoby *et al.*, 1976) and our results need not indicate increased activity of all; they could suggest increased activity in liver of only transferases A and E, and in kidney of only E. In lung, our finding of unchanged transferase activity indicates that the greater susceptibility of vitamin A-deficient respiratory tract to chemical carcinogens (Sporn *et al.*, 1976) is due to mechanisms other than the decreased glutathione *S*-transferase activity believed responsible elsewhere by Smith *et al.* (1977). However, in liver, the increased transferase activity we found may protect vitamin A-deficient rats against hepatocarcinogens metabolized there.

We thank Mr. Paul Honig and Miss Adrienne Dishman for their excellent technical assistance, Miss Susan T. Arnold for calculating the apparent enzyme kinetic parameters, and Mr. Bernard F. Taylor for maintaining the animals. The assistance of Miss Shaheen Siddik and Mrs. Afroz B. Siddik in preparing this manuscript is greatly appreciated.

References

Baars, A. J., Jansen, M. & Breimer, D. D. (1978) *Biochem. Pharmacol.* **27**, 2487–2494

Becking, G. C. (1973) *Can. J. Physiol. Pharmacol.* **51**, 6–11

Benson, A. M., Batzinger, R. P., Ou, S.-Y. L., Bueding, E., Cha, Y.-N. & Talalay, P. (1978) *Cancer Res.* **38**, 4486–4495

Chasseaud, L. F. (1976) in *Glutathione: Metabolism and Function* (Arias, I. M. & Jakoby, W. B., eds.), pp. 77–114, Raven Press, New York

Chhabra, R. S., Miranda, C. L., Bend, J. R., Mukhtar, H. & Zeiger, E. (1977) *Pharmacologist* **19**, 160

Clifton, G., Kaplowitz, N., Wallin, J. D. & Kuhlenkamp, J. (1975) *Biochem. J.* **150**, 259–262

Colby, H. D., Kramer, R. E., Greiner, J. W., Robinson, D. A., Krause, R. F. & Canady, W. J. (1975) *Biochem. Pharmacol.* **24**, 1644–1646

Dugan, R. E., Frigerio, N. A. & Siebert, J. M. (1964) *Anal. Chem.* **36**, 114–117

Fjellstedt, T. A., Allen, R. H., Duncan, B. K. & Jakoby, W. B. (1973) *J. Biol. Chem.* **248**, 3702–3707

Genta, V. M., Kaufman, D. G., Harris, C. C., Smith, J. M., Sporn, M. B. & Saffiotti, U. (1974) *Nature (London)* **247**, 48–49

Grover, P. L. & Sims, P. (1964) *Biochem. J.* **90**, 603–606

Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7130–7139

Jakoby, W. B., Habig, W. H., Keen, J. H., Ketley, J. N. & Pabst, M. J. (1976) in *Glutathione: Metabolism and Function* (Arias, I. M. & Jakoby, W. B., eds.), pp. 189–202, Raven Press, New York

Johnson, M. K. (1966) *Biochem. J.* **98**, 44–56

Kaplowitz, N., Kuhlenkamp, J. & Clifton, G. (1975) *Biochem. J.* **146**, 351–356

Knott, G. D. & Reese, D. K. (1972) in *Proc. On-line Int. Conf.* **1**, 497–526

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Moron, M. S., Depierre, J. W. & Mannervik, B. (1979) *Biochim. Biophys. Acta* **582**, 67–78

Siddik, Z. H., Mimnaugh, E. G., Trush, M. A. & Gram, T. E. (1979) *Proc. Int. Symp. Microsomes and Drug Oxidations 4th*, 68

Smith, B. R., Maguire, J. H., Ball, L. M. & Bend, J. R. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 2480–2484

Smith, G. J., Ohi, V. S. & Litwack, G. (1977) *Cancer Res.* **37**, 8–14

Sporn, M. B., Dunlop, N. M., Newton, D. L. & Smith, J. M. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1332–1338

Yonemoto, T. & Oh, M. (1969) *J. Vitaminol.* **15**, 254–260