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Comparison of Hepatic Glutathione S-Transferases of Male and Female Rats

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Six and eight peaks of activity toward 1-chloro-2,4-dinitrobenzene (CDNB) were observed in carboxymethyl (CM)-cellulose chromatograms of hepatic glutathione S-transferases in female and male rats, respectively. These peaks were named transferases I, II, IIIa, III, IIIb, IV, V and VI in the order of elution with KCl (transferases IIIa and IIIb could not be detected in the case of female rats). Transferase I could be further resolved into at least two enzymes by CM-Sepharose CL-6B chromatography. Transferase II was confirmed to consist of YaYa subunits (ligandin) by CM-cellulose chromatography; Habig *et al.*[1,2] did not detect this transferase because of their use of 1,2-dichloro-4-nitrobenzene (DCNB) as a substrate for assaying these species of transferases.

Phenobarbital treatment caused 43 and 93% increases in the total activity toward CDNB in female and male rat livers, respectively. Transferases I and II were strongly induced in both sexes of rats by this treatment. Transferase IV was slightly increased in both sexes. Transferases III and V were induced in male rats, but not in female rats.

The subunit species and substrate specificities of the present transferases were compared with those of reported transferases.

Keywords——glutathione; glutathione S-transferase; phenobarbital; induction; sex difference; subunit; ligandin

The glutathione S-transferases (EC 2.5.1.18) are abundant in rat liver cytosol, and have glutathione conjugating activity toward hydrophobic substrates bearing electrophilic groups,[1] as well as non-selenium-dependent glutathione peroxidase activity[3] and the ability to bind organic anions such as bilirubin,[4] steroid metabolites,[5,6] azo dye carcinogens,[7] *etc.*

The glutathione S-transferase activity has been shown to be higher in male rat liver than in the female.[8] Kaplowitz *et al.*[9] examined the sex difference in hepatic glutathione S-transferases by using 1,2-dichloro-4-dinitrobenzene (DCNB), *p*-nitrobenzyl chloride (*p*-NBC), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (ENPP) and methyl iodide as substrates, and higher activities toward DCNB, *p*-NBC and ENPP were observed in male rats. Moreover, it was found that transferase activities were induced in some organs by phenobarbital, 3-methylcholanthrene or benzo(α)pyrene.[8–12] Most of the previous studies on induction and sex difference of glutathione S-transferases have dealt with the levels of activities toward various substrates, without separating the enzymes involved. However, Habig *et al.*[1,2] and Fjellstedt *et al.*[13] separated seven glutathione S-transferases from rat liver by diethylaminoethyl (DEAE)- and carboxymethyl (CM)-cellulose column chromatography, and designated them as M, AA, A, B, C, D and E. They indicated these transferases to possess overlapping substrate specificities. We have also carried out the separation of glutathione S-transferases, and investigated the sex difference in the effects of phenobarbital on individual glutathione S-transferases. The results are described in the present paper.

Experimental

Materials——Male (*ca.* 350 g) and female (*ca.* 250 g) Wistar rats were purchased from Sankyo Labo. Service

Corp. DEAE (DE-23, 1.0±0.1 meq/g) and CM cellulose (CM-52, 1.0±0.1 meq/g) were obtained from Whatman Chemical Separation. Sephadex G-75 (superfine) and CM-Sepharose CL-6B (12±2 meq/100 ml gel bed) were from Pharmacia Fine Chemicals. Hydroxylapatite was prepared by the method of Tiselius *et al.*[14] Benzalacetone (BA), *p*-NBC, DCNB and sodium dodecyl sulfate (SDS) were from Wako Pure Chemical Ind. 1-Chloro-2,4-dinitrobenzene (CDNB) and ENPP were from Tokyo Kasei Kogyo Co., and Eastman Kodak Co., respectively. Ethacrynic acid (EA) was obtaind from Sigma Chemical Co. Other chemicals were of commercial guaranteed grade.

Protein Concentration——This was estimated by measuring the absorbance at 280 nm or by the method of Lowry *et al.*[15] with bovine serum albumin as a standard in a quartz cell of 1 cm light path on a Hitachi model 100-60 spectrophotometer.

Enzyme Assay——The glutathione S-transferase activity toward various electrophilic compounds was spectrophotometrically measured at 25 °C. The concentrations of substrates, the wavelength, pH and the molar extinction coefficients were adopted from the paper of Habig *et al.*[1] except for the wavelength (320 nm) and the molar extinction coefficient (1.75 mM^{-1} cm^{-1}) of *p*-NBC.

SDS/Polyacrylamide-Gel Electrophoresis——This was carried out in the presence of 0.1% (w/v) SDS according to the method of Laemmli.[16] The stacking gel slab (2.5×13.5×0.2 cm) consisted of a 3% (w/v) gel in 0.125 M Tris-HCl buffer, pH 6.8. The resolving gel slab (10×13.5×0.2 cm) consisted of a 12.5% (w/v) gel in 0.375 M Tris-HCl buffer, pH 8.8. The electrode buffer was 0.025 M Tris-glycine buffer, pH 8.3, containing 0.1% (w/v) SDS. Samples were pre-treated by heating for 5 min at 90 °C in 10 mM Tris-HCl buffer, pH 6.8, containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 20% (v/v) glycerol. Electrophoresis was performed for 16 h at a constant current of 10 mA. The gel slab stained with 0.025% (w/v) Coomassie Brilliant Blue R-250 in an aqueous solution containing 50% (v/v) methanol and 10% (v/v) acetic acid. Excess color was removed by diffusion in an aqueous solution containing 25% (v/v) methanol and 7% (v/v) acetic acid.

Separation of Hepatic Glutathione S-Transferases by CM-Cellulose Column Chromatography——This was essentially performed at 4 °C by the method of Habig *et al.*[1] Male or female rats were sacrificed by decapitation. The livers were cut into small pieces and homogenized with distilled water (5 ml per g wet liver) in a Teflon-glass homogenizer. The homogenate was centrifuged at 20000×*g* for 1 h. The resulting supernatant was adjusted to pH 8.0 with 1 M Tris base. Supernatant equivalent to 20 g wet liver was applied to a DEAE-cellulose column (2.8×32 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The column was washed with the above buffer until the first group of transferases had been eluted. The active fractions were combined in a cellophane tube (type 36/32 inch) and concentrated by dialysis against polyethylene glycol 20000. The concentrated preparation (*ca.* 10 ml) was dialyzed for 1 d against three changes, each of 1 l, of 10 mM potassium phosphate buffer, pH 6.7, and charged onto a CM-cellulose column (1.5×13 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.7. The column was washed with 120 ml of the above buffer and the adsorbed proteins were eluted with a linear gradient of 0 to 80 mM KCl in the above buffer (400 ml).

Induction of Glutathione S-Transferases by Phenobarbital Treatment——Phenobarbital was orally administered to three male and three female rats at a dose of 80 mg/kg daily for 7 d. The rats were decapitated 24 h after the last dose. Twenty grams of livers from rats of each sex was subjected to the separation procedure described above.

Results

Glutathione S-Transferases in Male and Female Rat Livers

The activities toward CDNB of normal male and female rat livers were 79 and 96 μmol/min/g wet weight, respectively. Phenobarbital treatment caused an increase of 93% in hepatic

TABLE I. Glutathione S-Transferase Activities of Male and Female Rats

Preparation	Male		Female	
	Control	Phenobarbital treatment	Control	Phenobarbital treatment
20000×*g* supernatant	1538	2963	1388	1988
DEAE-cellulose				
Adsorbed	160	152	118	110
Non-adsorbed	899	2286	804	1750

These values (μmol/min) of activity toward CDNB were obtained with 20 g wet livers.

glutathione S-transferase activity for male rats and an increase of 43% for the females. These increases of activity were attributable to glutathione S-transferases which passed through a DEAE-cellulose column. The activities of DEAE-non-adsorbed fractions were increased from 899 to 2286 μmol/min for male rats by phenobarbital treatment and from 804 to 1750 μmol/min for the females, while no increase of DEAE-adsorbed activity was observed for either sex (Table I).

CM-Cellulose Chromatographic Patterns of Glutathione S-Transferases

Non-adsorbed fractions on DEAE-cellulose were further chromatographed on a CM-cellulose column, and then the activity was assayed by using glutathione and CDNB or

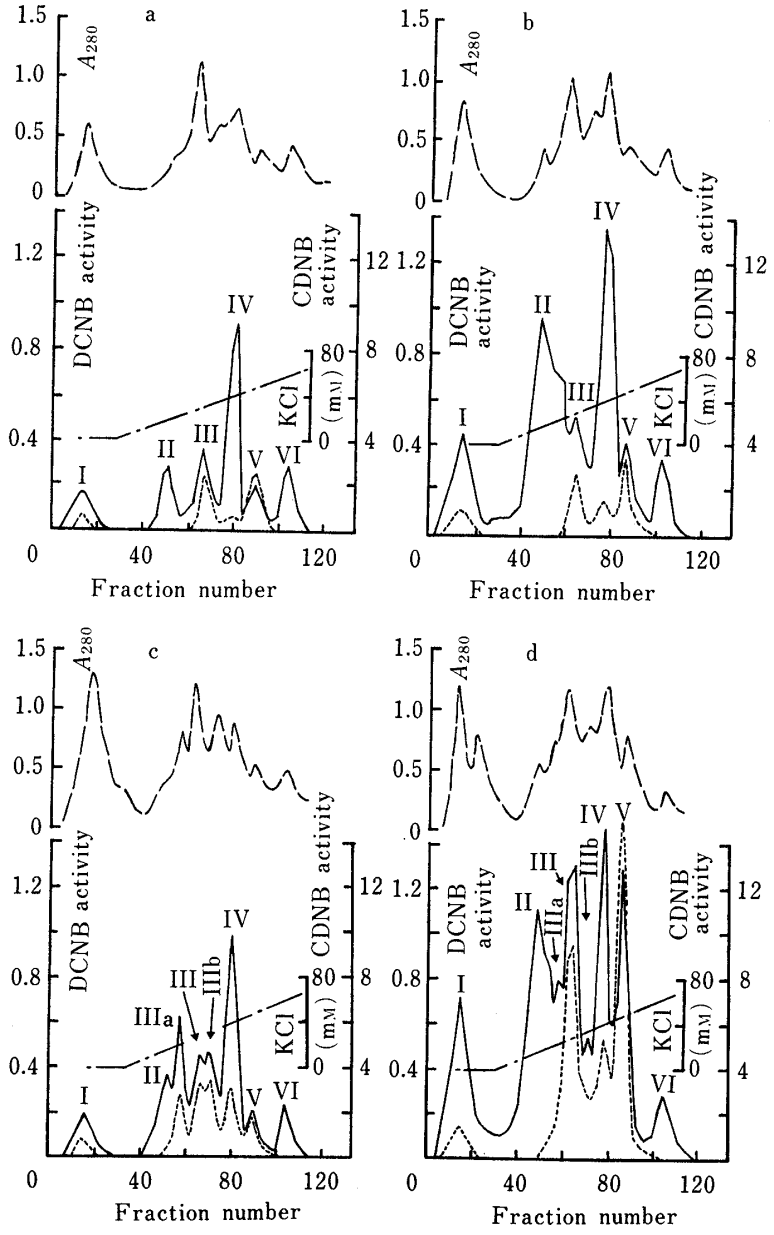

Fig. 1-a, b, c, d. Chromatography of Hepatic Glutathione S-Transferases on a CM-Cellulose Column

Fractions of 4 ml were collected. a, female normal rats; b, female rats treated with phenobarbital; c, male normal rats; d, male rats treated with phenobarbital. ----, A_{280}; ------, DCNB activity (μmol/min/ml); ——, CDNB activity (μmol/min/ml); —·—, KCl concentration. See the text for experimental details.

DCNB. Both phenobarbital-treated and non-treated female rats (Fig. 1-a, b) showed six peaks of glutathione S-transferase, able to conjugate glutathione with CDNB, eluted at concentrations of 0, 15, 28, 40, 48 and 62 mM KCl. These activity peaks were named transferases I, II, III, IV, V and VI in the order of elution. However, when DCNB was used as a substrate in place of CDNB, transferases II and VI were almost undetectable. The activities of transferases I and II toward CDNB were increased significantly by phenobarbital treatment and that of transferase IV was increased slightly, but the activities of transferases III, V and VI were unaffected. In male rats, two additional activity peaks appeared at concentrations of 20 mM KCl for transferase IIIa and 34 mM KCl for transferase IIIb (Fig. 1-c, d). Moreover, a clear difference in the effect of phenobarbital was found between male and female rats. That is to say, transferases I, II, and IV were induced in both sexes, but transferases III and V were induced only in the case of male rats. The increase of activity toward DCNB was greater for male rats than the females, because transferases III and V each possessed high specific activity toward DCNB.

Subunit Components

Transferases AA, A, B and C, as proposed by Habig *et al.*,[1] have been identified as consisting of YcYc, Yb_1Yb_1, YaYc and Yb_1Yb_2 subunits (Ya, mol. wt. 22000; $Yb(Yb_1, Yb_2)$, mol. wt. 23500; Yc, mol. wt. 25000),[17-20] respectively. Therefore, the subunit species of our glutathione S-transferases were also examined by discontinuous SDS/polyacrylamide-gel electrophoresis of the fractions (Fig. 1-a, c) obtained from the CM-cellulose column. The electrophoretic patterns (Fig. 2-a, b) showed the lanes of transferases I, II, IIIa, III, IIIb, IV, V and VI to have mainly Ya-Yb, Ya, Ya-Yb, Yb, Ya-Yb-Yc, Ya-Yb-Yc, Yb-Yc and Yc subunit components, respectively. On the basis of the subunit components and the order of elution from the CM-cellulose column, transferases I, III, IV, V and VI were considered to be identical with transferases D and E, C, B, A and AA, respectively. Phenobarbital treatment resulted in a remarkable increase in the activity of transferase II, which comprised only the Ya subunit component. Transferase II also possessed the ability to bind bromosulfophthalein (data not shown). From these results, this transferase II was considered to correspond to authentic transferase YaYa.[17]

We found two new activity peaks, transferases IIIa and IIIb, which have not been clearly described by any other worker. However, we could not clarify their physico-chemical properties, because four transferases (II, IIIa, III and IIIb) often appeared in one or two activity peaks on the CM-cellulose chromatogram.

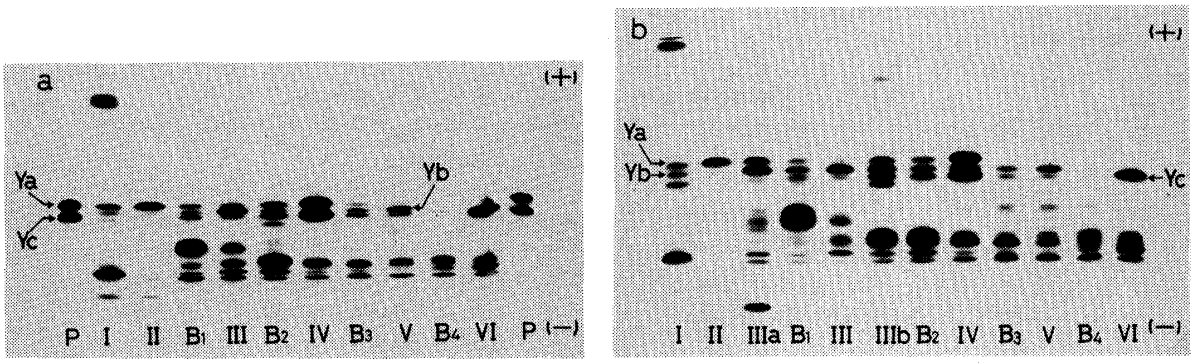

Fig. 2-a, b. SDS/Polyacrylamide-Gel Electrophoretic Patterns of the Fractions from CM-Cellulose Chromatography

A portion (50 μl) of each fraction was loaded onto polyacrylamide gel. a, female rats. P, purified transferase IV (43 μg); I, No. 13; II, No. 49; B_1, No. 59; III, No. 64; B_2, No. 72; IV, No. 78; B_3, No. 84; V, No. 88; B_4, No. 98; VI, No. 104 from Fig. 1-a. b, male rats. I, No. 14; II, No. 52; IIIa, No. 58; B_1, No. 62; III, No. 66; IIIb, No. 72; B_2, No. 76; IV, No. 80; B_3, No. 86; V, No. 90; B_4, No. 100; VI, No. 106 from Fig. 1-c. See the text for experimental details.

Preparation of Glutathione S-Transferases

Separation of glutathione S-transferases was performed on a large scale and transferases I—VI were further purified on Sephadex G-75 and hydroxylapatite columns. Transferase I was charged onto a Sepharose CL-6B column (2.7 × 21 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.0, prior to additional separation procedures. The elution was performed with a KCl linear gradient of 0 to 300 mM in the above buffer (400 ml) (Fig. 3). Two activity peaks appeared at 80 and 130 mM KCl, and were named transferases Ia and Ib, respectively.

The finally purified transferases were confirmed to be homogenous or nearly homogenous by SDS/polyacrylamide-gel electrophoresis (Fig. 4). Although a faint Yb-subunit band was observed in the electrophoretic patterns of transferase I (Fig. 2-a, b), the purified transferases Ia and Ib resolved from transferase I both had YaYa subunits, as did transferase II. Transferases III and V had YbYb subunits, but it was not determined whether the Yb subunit component was Yb_1 or Yb_2 in the present study. Transferases IV and VI had YaYc and YcYc subunits, respectively. Although transferases IIIa and IIIb were obtained in only small amounts, they appeared to consist of YbYb subunits.

Substrate Specificity

Table II lists the substrate specificities of various transferases. CDNB was the most

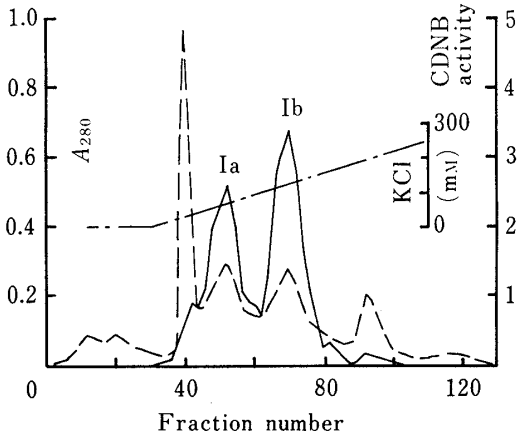

Fig. 3. Chromatography of Transferase I on a CM-Sepharose CL-6B Column

Fractions of 4 ml were collected. ----, A_{280}; ——, CDNB activity (μmol/min/ml); — - —, KCl concentration.

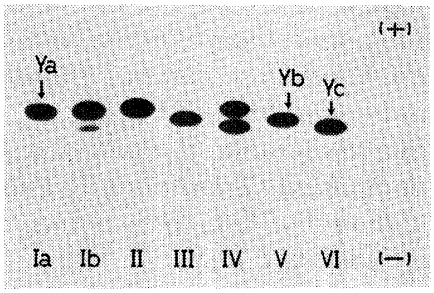

Fig. 4. SDS/Polyacrylamide-Gel Electrophoretic Pattern of the Finally Purified Transferases

Ia, 46 μg; Ib, 50 μg; II, 39 μg; III, 32 μg; IV, 43 μg; V, 37 μg; VI, 43 μg. Transferases Ia, Ib and II each showed a small amount of contaminant at the position of the Yc-subunit band.

Table II. Substrate Specificities of Various Transferases

Transferase	Subunit	Substrate					
		CDNB	DCNB	*p*-NBC	BA	EA	ENPP
Ia	YaYa	18	0.007	0.29	0.001	0.19	0.04
Ib	YaYa	17	0.006	0.29	0.004	0.27	0.08
II	YaYa	22	0.010	0.59	0.004	0.25	0.05
III[a)]	Yb_1Yb_2	35	1.8	16	0.75	0.2	0.43
IV	YaYc	18	0.005	0.28	0.003	0.44	0.02
V[a)]	Yb_1Yb_1	54	3.4	16	0.073	0.15	0.25
VI	YcYc	9	0.017	0.27	0.004	0.99	0.04

These values are μmol/min/mg.
a) Data for the subunits of transferases III and V are from Hayes.[20)]

suitable substrate among those examined for the purpose of detecting all the transferases. Transferases Ia, Ib and II comprising YaYa subunits possessed similar substrate specificities, so that only their chromatographic behavior could distinguish them. The substrate specificities of transferases IV (YaYc subunits) and VI (YcYc subunits) were similar to those of the above three transferases in that they had low specific activities toward DCNB, *p*-NBC, BA and ENPP. The specific activities toward EA were in the order YaYa, Yb_1Yb_1, Yb_1Yb_2 < YaYc < YcYc subunits. Transferases III and V, comprising Yb_1Yb_2 and Yb_1Yb_1 subunits, respectively, had relatively high specific activities toward DCNB, *p*-NBC and ENPP. They were distinguishable since the specific activity of transferase III toward BA was 10 times that of transferase V.

Discussion

The glutathione S-transferase activity toward CDNB in male rat liver was shown to be increased much more significantly by phenobarbital treatment than that in females. Since phenobarbital treatment did not affect the activities of DEAE-adsorbed fractions from either sex, and induced transferases I and II similarly, the difference in extent of induction appeared to depend on the inducibility of transferases III and V, which could be induced only in male rats. It has been reported that transferase A is induced by phenobarbital treatment of male rats, but transferase C is not.[21-23] However, our data showed increases of both transferases III (C) and V (A). The differences in induction and species of hepatic glutathione S-transferases observed between the sexes may be related to the hormonal status of the animal.

Transferase I seemed to correspond to transferases D and E in elution position from a CM-cellulose column, and it could be resolved into two activity peaks toward CDNB, transferases Ia and Ib consisting of YaYa subunits, by CM-Sepharose CL-6B column chromatography. Transferases Ia and Ib both had a low specific activity toward ENPP (Table II). This result conflicts with previous reports in that transferase E possessed YbYb subunits[24] and higher specific activity toward ENPP than any other transferase.[1,13] Hence, transferase Ia or Ib seemed to be distinct from transferase E. However, it is possible that transferase E in the same fraction as transferase I is lost by denaturation during the purification, because a partially purified transferase I obtained before the step of CM-Sepharose CL-6B column chromatography showed a faint Yb-subunit band in the electrophoretic patterns (Fig. 2-a, b). Recently, Hayes[20] indicated that transferase E existed in one of three activity peaks toward CDNB that were eluted from a DEAE-cellulose column with a 0—105 mM NaCl gradient. Moreover, transferase D was demonstrated to exist in the DEAE-non-adsorbed fraction and to consist of Yb_2Yb_2 subunits on the basis of hybridization, immunotitration, substrate specificity and peptide "mapping" experiments. Therefore, in our experiment transferase possessing a high specific activity toward ENPP might have been adsorbed by DEAE-cellulose. No transferase corresponding to transferase D could be found in the DEAE-non-adsorbed fraction. Transferases III and V, corresponding to transferases C and A, respectively, both had a high ability to conjugate glutathione with ENPP. A similar result was obtained when the CM-cellulose column fractions were examined by our activity-monitoring method permitting direct measurement of the glutathione-ENPP conjugate.[25] These values of specific activity were higher than those of Habig *et al.*,[1] but similar to those of Hayes.[20]

It is known that ligandin can bind a number of compounds having hydrophobic moieties which are not utilized as substrates. In addition, a report[26] suggested that ligandin was identical with transferase B with respect to substrate specificity, binding ability and immunological properties. However, confusion exists as to whether ligandin consists of YaYa,[21] YaYc subunits (transferase B)[27] or a mixture of two enzymes.[28] We found

transferase II (ligandin) to be similar to transferase B in terms of the substrate specificity, but different in terms of the subunit components. It had YaYa subunits, and could be eluted as a peak between transferases D and E, and C on CM-cellulose column chromatography. Although transferases Ia, Ib and II all consist of YaYa subunits and possess similar substrate specificities, it is unclear whether these three types arise in the same manner as Yb_1 and Yb_2 subunit components, or by an *in vivo* deamidation as assumed in the case of human hepatic glutathione S-transferases.[29]

Peaks of glutathione S-transferases IIIa and IIIb were recognized only in male rats, whether given phenobarbital treatment or not, but the relative amounts showed poor reproducibility. Transferases II, IIIa, III and IIIb were often combined in one or two peaks. Therefore, it is not clear whether transferases IIIa and IIIb exist exclusively in the male rat liver or not. Hayes and Clarkson[30] reported that transferase A (V) was further resolved into three peaks in hydroxylapatite chromatography by employing a gentler gradient elution system than that used by Habig *et al.*,[1] and reported that these three types arose as a result of autoxidation. Although our transferases IIIa and IIIb were not examined for autoxidation, they may be modified forms of transferases C (III) and/or A (V) because our transferases possess considerable activity toward DCNB and may have YbYb subunit components.

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