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Further Analysis of Multiple Forms of Rabbit Hepatic Glutathione S-Transferase

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In the previous study,¹⁾ carboxymethyl (CM)-cellulose chromatography of a rabbit liver extract gave at least four glutathione S-transferase activity peaks (peaks R1, R2, R3 and R4). Further purification of peaks R2 and R3 by S-hexylglutathione Sepharose 6B and hydroxylapatite chromatography resolved peak R2 into one peak (a homodimer of the Y2Y2 subunits; Y2 M_r 25000), and peak R3 into two peaks, R3a (a heterodimer of the Y1Y3 subunits; Y1 M_r 24500; Y3 M_r 26500) and R3b (a homodimer of the Y3Y3 subunits). In the present study, isoelectric focusing was used to separate the diethylaminoethyl (DEAE)-flow-through fraction into at least eight activity peaks (pIs 10.95–10.77, 10.35, 9.95, 9.40, 8.88, 8.48, 8.36 and 7.66), which were designated as activity peaks I, II, III, IV, V, VI, VII and VIII in decreasing order of isoelectric point. On the other hand, these peaks could be resolved into at least seven activity peaks by conventional CM-cellulose chromatography, the four main activity peaks of which were assigned as peaks R1, R2, R3 and R4 reported previously.¹⁾ As a result of the individual isoelectric focusing of peaks R1–R4, activity peak I having the highest and broadest isoelectric point was identified as peak R4. By sodium dodecyl sulfate (SDS)/polyacrylamide-gel electrophoresis, peaks II, III and VII were identified as the previously characterized R3a, R3b and R2, respectively. The isoelectric focusing of peak R1 gave four activity peaks (pIs 9.72, 8.56, 7.64 and 7.07). The enzyme with pI 9.72 was found to be a homodimer of the Y1Y1 subunits and the other three enzymes were all heterodimers of Y2 subunit and a new type of subunit (designated as Y4; M_r 28000). Among these four peaks, the enzymes with pIs 9.72 and 7.07 could not be observed in the isoelectric focusing pattern of the gross DEAE-flow-through fraction. The enzymes with pIs 8.56 and 7.64 were identified as peaks VI and VIII, respectively, on the basis of SDS/polyacrylamide-gel electrophoresis and their isoelectric points.

Keywords—glutathione; glutathione S-transferase; subunit; rabbit liver; isoelectric focusing

Glutathione S-transferases (EC 2.5.1.18) are a family of enzymes which play an important role in the detoxication and excretion of xenobiotics.²⁾ These enzymes are present in various species and tissues. The existence of the multiple forms of glutathione S-transferase has been observed in rat,³⁾ human,⁴⁾ and monkey⁵⁾ livers, *etc.* They have molecular weights of 40000–50000 and consist of two subunits, and most of them have basic isoelectric points.

Although the rabbit is frequently used in *in vivo* experiments on the metabolism of xenobiotics, only a few reports^{1,6)} have appeared on the glutathione S-transferases from rabbit. Previously, we showed the presence of at least four activity peaks in the diethylaminoethyl (DEAE)-cellulose-flow-through fraction on carboxymethyl (CM)-cellulose chromatography.¹⁾ They were named peaks R1, R2, R3 and R4 in order of elution. The main peaks, R2 and R3, were purified to homogeneity as judged by SDS/polyacrylamide-gel electrophoresis. Peak R2 was found to be a homodimer of subunits with the molecular weight of 25000 (Y2). Peak R3 contained two forms, which were named R3a and R3b in order of elution from a hydroxylapatite column. R3a was a heterodimer of subunits with molecular weights of 24500 (Y1) and 26500 (Y3), and R3b was a homodimer of subunits with the molecular weight of 26500 (Y3). However, the above hydroxylapatite column

chromatography could not clearly determine the number of multiple forms in each of peaks R1 and R4 (unpublished results). Therefore, we further studied the multiple forms of glutathione S-transferase by separately subjecting the DEAE-flow-through fraction and peaks R1, R2, R3 and R4 obtained after CM-cellulose chromatography to isoelectric focusing in a column.

Experimental

Materials—Female Japanese white rabbits (body weight, about 3.8 kg) were purchased from Sankyo Labo. and fed a standard diet (100 g/head/d, Clea Japan Inc.) and tap water *ad libitum*. DEAE (DE-23, 1.0 ± 0.1 meq/g)- and CM (CM-52, 1.0 ± 0.1 meq/g)-cellulose were obtained from Whatman Chemical Separation Ltd. Sephadex G-75 (superfine), Sepharose 6B and Pharmalyte (pH 10.5–8 and pH 9–6.5) were from Pharmacia Fine Chemicals. Epoxy-activated Sepharose 6B was prepared as described by Sundberg and Porath.⁷⁾ S-Hexylglutathione was synthesized by the procedure of Vince *et al.*⁸⁾ The coupling of S-hexylglutathione and epoxy-activated Sepharose 6B was carried out according to the previous report.¹⁾ 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Wako Pure Chemical Ind., Ltd. All other chemicals were of the highest purity available.

Enzyme Assay—Enzyme activity was spectrophotometrically determined by measuring the rate of conjugation of glutathione (GSH) with CDNB according to the report of Habig *et al.*³⁾ One unit of enzyme activity was defined as the amount of enzyme conjugating 1 μ mol of substrate per min at 25 °C.

Protein Concentration—This was determined by measurement of the absorbance at 280 nm in a quartz cell of 1 cm light path using a Shimadzu model UV-240 spectrophotometer.

Separation of Glutathione S-Transferases—This was performed at 4 °C by the method described in the previous report.¹⁾

1. Extraction: A female rabbit was sacrificed by injecting 10 ml of air into an ear vein. The liver (121.5 g) was removed, perfused with 0.25 M sucrose, 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 $20000 \times g$ for 1 h. The supernatant was adjusted to pH 8.0 with 1 M Tris base.

2. Passage through a DEAE-Cellulose Column: The above crude extract was applied to a DEAE-cellulose column (5.5×50 cm) equilibrated with 10 mM Tris-HCl, pH 8.0. The column was washed with 2.5 l of the above buffer and eluted with 2 l of the same buffer containing 0.5 M KCl. Most of the activity toward CDNB was recovered in the flow-through fraction. This fraction was packed in cellophane tubing (type 36/32 inch) and concentrated by dialysis against polyethylene glycol 20000. The concentrated sample was dialyzed against four changes, each of 4 l, of 10 mM potassium phosphate buffer, pH 6.7, for 8 h. Insoluble materials precipitated during dialysis were removed by centrifugation at $20000 \times g$ for 15 min. A portion (10 ml) of the supernatant (56 ml) was applied to a Sephadex G-75 column (2.7×92 cm) equilibrated with 1 mM potassium phosphate buffer, pH 6.7, and only one peak of enzyme activity was eluted, corresponding to a molecular weight of approximately 51000. Active fractions were combined and subjected to isoelectric focusing in a column.

3. CM-Cellulose Chromatography: The remaining supernatant (46 ml) from step 2 was applied to a CM-cellulose column (2.8×30 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.7. After being washed with 560 ml of the above buffer, the column was eluted with a linear gradient of 0 to 60 mM KCl in the same buffer (2 l). At least seven activity peaks were observed and four of them were found to be peaks R1, R2, R3 and R4 reported previously.¹⁾ These four fractions were separately packed in cellophane tubing and concentrated by dialysis against polyethylene glycol 20000. The concentrated samples were dialyzed against four changes, each of 4 l, of 1 mM potassium phosphate buffer, pH 6.7, for 8 h. Insoluble materials precipitated during dialysis were removed by centrifugation at $20000 \times g$ for 15 min. Each supernatant was subjected to isoelectric focusing in a 110 ml column.

4. Isoelectric Focusing in Column: A mixture of Pharmalytes with pH ranges of 10.5–8 and 9–6.5 (3:1, v/v), as carrier ampholytes, was used at 1% final concentration. Amounts of protein and the enzyme activity of each sample used are summarized in Table I. Isoelectric focusing was performed in a 0–50% sorbitol density gradient at 3 W for 46 h at 2 °C with a 110 ml column. Fractions of 1.5 ml were collected and assayed for glutathione S-transferase activity. The pH of each fraction was measured at 2 °C immediately after collection.

Affinity Chromatography—Each activity peak obtained by isoelectric focusing was separately dialyzed against three changes, each of 5 l, of 10 mM Tris-HCl, pH 8.0, for 24 h. The dialyzed samples were applied to small affinity columns (column vol., 0.5 ml). The columns were washed with 3 ml of the above buffer containing 0.2 M KCl and then eluted with 1.5 ml of the same buffer containing 5 mM S-hexylglutathione, 2.5 mM glutathione and 0.2 M KCl. The effluents were subjected to SDS/polyacrylamide-gel electrophoresis.

SDS/Polyacrylamide-Gel Electrophoresis—This was carried out in the presence of 0.1% (w/v) SDS according to the method of Laemmli⁹⁾ with 10% polyacrylamide slab gel. Samples were pre-treated by heating for 5 min at 90 °C with equal volumes of 20 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 40% (v/v)

TABLE I. Samples for Isoelectric Focusing

Sample	Total		Specific activity ^{c)}
	Protein ^{a)}	Activity ^{b)}	
Mixture ^{d)}	31	1150	37
R1	12.8	406	32
R2	11	555	51
R3	11.9	858	72
R4	17.5	230	13

a) Absorbance at 280 nm. b) $\mu\text{mol}/\text{min}$ (CDNB as a substrate). c) $\mu\text{mol}/\text{min}/A_{280}$. d) DEAE-cellulose-flow-through fraction. R1—4: Each sample was obtained from CM-cellulose chromatography.

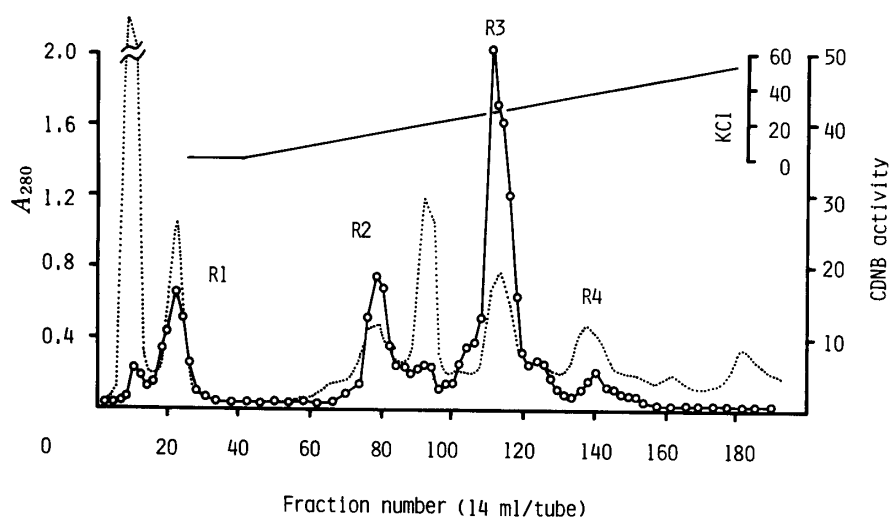


Fig. 1. Chromatography of Glutathione S-Transferases from Rabbit Liver on a CM-Cellulose Column

-----, A_{280} ; —○—, CDNB activity ($\mu\text{mol}/\text{min}/\text{ml}$); —, KCl concentration (mM). For details, see Experimental.

glycerol. Each sample (4—8 μg) was electrophoresed for 16 h at a constant current of 8 mA. After electrophoresis, the gel was stained with 0.025% (w/v) Coomassie Brilliant Blue R-250 in a solvent mixture of methanol, acetic acid and water (50:10:40, v/v) and destained in a solvent mixture of methanol, acetic acid and water (25:7:68, v/v).

Results

Separation of Glutathione S-Transferases by CM-Cellulose Column Chromatography

Rabbit liver showed an activity of 210 $\mu\text{mol}/\text{min}/\text{g}$ wet tissue toward CDNB. Most of the activity in the 20000 $\times g$ supernatant was recovered in the DEAE-flow-through fraction. Only a few percent of the activity in the above supernatant was bound to the column (result not shown). When the flow-through fraction was applied to a CM-cellulose column and eluted with a linear gradient of 0—60 mM KCl, this flow-through fraction was resolved into at least seven activity peaks (Fig. 1). The four main peaks among them corresponded to the previously reported peaks R1, R2, R3 and R4 as judged from the KCl concentrations at which these peaks were eluted. The other three peaks were newly observed in the present study.

Isoelectric Focusing in a Column

DEAE-Flow-through Fraction—As shown in Fig. 2, the isoelectric focusing of the

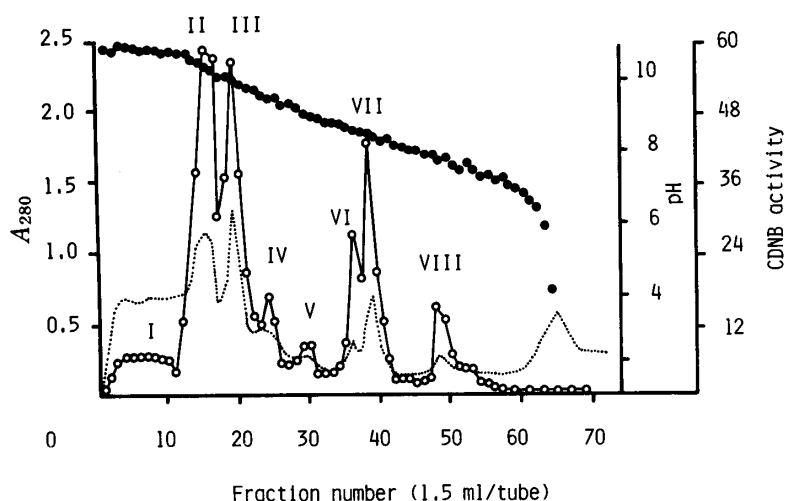


Fig. 2. Isoelectric Focusing of DEAE-Flow-through Fraction

----, A_{280} ; —○—, CDNB activity ($\mu\text{mol}/\text{min}/\text{ml}$); ●●●, pH. For details, see Experimental.

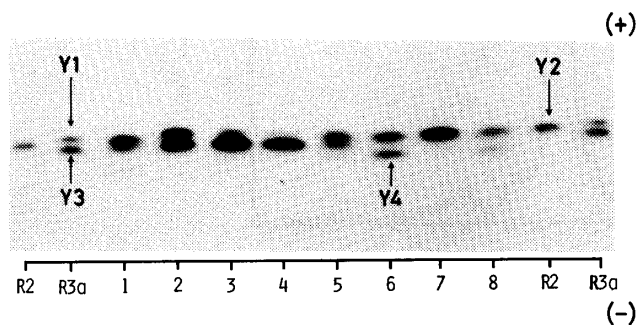


Fig. 3. SDS/Polyacrylamide-Gel Electrophoretic Pattern

R2 and R3a, glutathione S-transferases purified from rabbit liver according to the previous method¹¹; 1, a mixture of No. 4—8; 2, No. 15; 3, No. 19; 4, No. 24; 5, No. 30; 6, No. 36; 7, No. 38; 8, No. 48 from Fig. 2.

DEAE-flow-through fraction gave at least eight activity peaks, and these were focused at pHs of 10.95—10.77, 10.35, 9.95, 9.40, 8.88, 8.48, 8.36 and 7.66. These activity peaks were designated as activity peaks I, II, III, IV, V, VI, VII and VIII in decreasing order of isoelectric point. By SDS/polyacrylamide-gel electrophoresis as shown in Fig. 3, it was found that peak II was composed of the Y1Y3 subunits. Peak III was found to be composed of the Y3Y3 subunits, though it was slightly contaminated by the Y1 subunit of peak II. Peak IV was found to have the Y3Y3 subunits, though it was slightly contaminated by the Y1 subunit of peak II. Peak VI seemed to be a heterodimer of Y2 subunit and a new type of subunit. This new type of subunit was designated as Y4. Peak VII was found to be a dimer of the Y2Y2 subunits. The subunit compositions of the other peaks were unclear.

Peaks R1—R4—Peaks R1—R4 obtained by CM-cellulose chromatography were separately subjected to isoelectric focusing in a column. The isoelectric focusing patterns of peaks R1—R4 are shown in Fig. 4, respectively. Peak R1 was resolved into four activity peaks, and these were focused at the pHs of 9.72, 8.56, 7.64 and 7.07 (Fig. 4a). The activity peak with pI 9.72 had the Y1Y1 subunits (Fig. 5a). This peak was found to be different from either of peaks III (pI 9.95) IV (pI 9.40) in Fig. 2, both of which were dimers of the Y3Y3 subunits. All of the other three peaks (Fig. 5a: lane Nos. 2, 3 and 4) were composed of Y2 subunit and a new type of subunit (Y4). The molecular weight of Y4 subunit was determined to be 28000 by SDS/polyacrylamide-gel electrophoresis using the following standard marker proteins (data not shown): bovine serum albumin (M_r 66000), ovalbumin (45000), carbonic anhydrase (29000), α -chymotrypsinogen-A (25000), myoglobin (17200), cytochrome c (12400) and the previously characterized rat hepatic glutathione S-transferases (Ya 24000, Yb 25500, Yc 27500).^{1,10} Under these conditions, Y1, Y2 and Y3 subunits showed molecular weights of

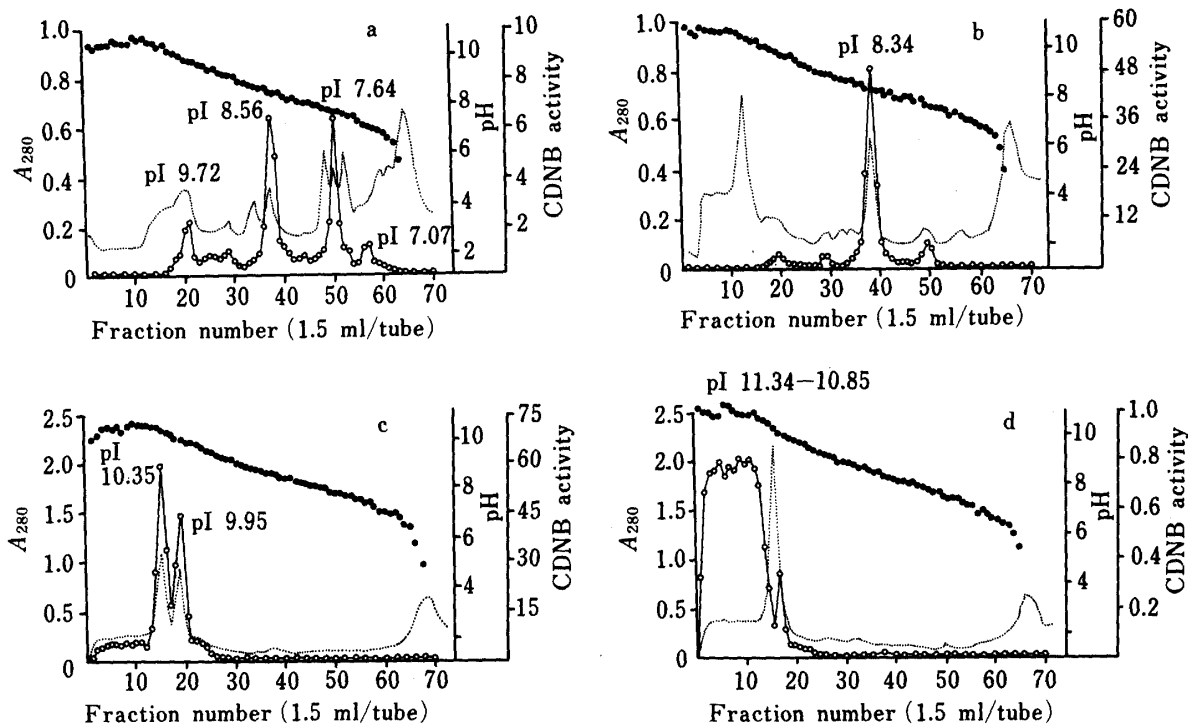


Fig. 4. Isoelectric Focusing of Peaks R1—R4 from CM-Cellulose Column Chromatography

a, peak R1. b, peak R2. c, peak R3. d, peak R4. ----, A_{280} ; —○—, CDNB activity ($\mu\text{mol}/\text{min}/\text{ml}$); ●●●, pH. For details, see Experimental.

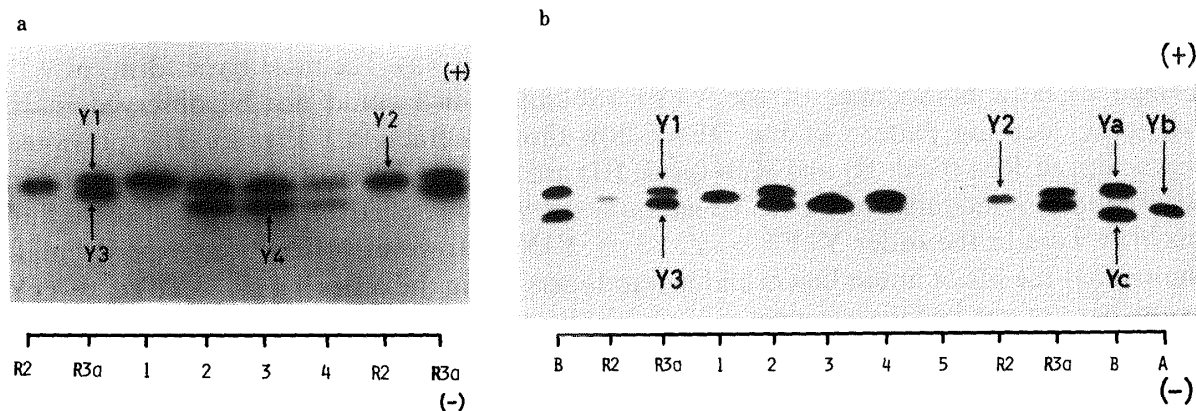


Fig. 5. SDS/Polyacrylamide-Gel Electrophoretic Patterns of the Fractions from Isoelectric Focusing of Peaks R1—R4

a, peak R1. R2 and R3a, glutathione S-transferases purified from rabbit liver according to the previous method¹¹; 1, No. 21; 2, No. 37; 3, No. 50; 4, No. 57 from Fig. 4a. b, peaks R2—R4. R2 and R3a, the purified glutathione S-transferases; A and B, glutathione S-transferases purified from rat liver according to the previous method¹⁰; 1, No. 38 from Fig. 4b; 2, No. 15; 3, No. 19 from Fig. 4c; 4, a mixture of No. 3—11; 5, No. 17 from Fig. 4d.

24500, 25000 and 26500, respectively. These values are identical with those of the subunits obtained in the previous studies.¹¹ The peaks having pI 8.56 and 7.64 in Fig. 4a were identified as peaks VI (pI 8.48) and VIII (pI 7.66) in Fig. 2, respectively. However, the Y2 content in peak VIII seemed to be increased as a result of contamination with Y2 subunit of peak VII. The activity peak with pI 7.07 (Fig. 4a) could not be identified by comparison with any of the peaks in Fig. 2. Peak R2 (Fig. 4b) contained one main activity peak, and this was composed of the Y2Y2 subunits as shown in lane 1 of Fig. 5b. Moreover, this peak had a pI value of 8.34

and was identical with peak VII (pI 8.36) in Fig. 2. Peak R3 (Fig. 4c) contained two activity peaks with pI 10.35 and 9.95, which were composed of the Y1Y3 and Y3Y3 subunits as shown in lanes 2 and 3 of Fig. 5b, respectively. These two peaks were found to be identical with peaks II (pI 10.35) and III (pI 9.95) in Fig. 2. Peak R4 having the highest pI value may contain several multiple forms in view of the broad pI value (Fig. 4d) and different amounts of Y2 subunit and Y3 subunit as shown in lane 4 of Fig. 5b.

Discussion

We have previously reported that the activity toward CDNB in female rabbit livers varied markedly from 44 to 362 $\mu\text{mol}/\text{min}/\text{g}$ wet tissue and that the relative intensities of activity peaks R1—R4 obtained by CM-cellulose column chromatography varied considerably from rabbit to rabbit.¹⁾ These variations were thought to be due to individual differences. Rabbit liver used in this study showed a high activity of 210 $\mu\text{mol}/\text{min}/\text{g}$ wet tissue and gave all of the activity peaks R1—R4 which have been previously reported.¹⁾

When the DEAE-flow-through fraction was applied to a CM-cellulose column and eluted with a linear gradient of 0—60 mM KCl, this fraction gave at least seven activity peaks. Four of them were found to be identical with peaks R1, R2, R3 and R4 as judged from their SDS/polyacrylamide-gel electrophoretic patterns (data not shown) and the KCl concentrations required to elute them. The DEAE-flow-through fraction, which contained all the basic transferases, was subjected to isoelectric focusing in a column after being deionized by Sephadex G-75 gel filtration. This isoelectric focusing showed the existence of at least eight forms of glutathione S-transferase. To compare the enzymes obtained by the isoelectric focusing with those in peaks R1—R4 obtained by CM-cellulose chromatography, peaks R1—R4 were separately subjected to isoelectric focusing. The relationships can be summarized as follows: I (R4), II (R3a), III (R3b), IV and V (not identified), VI (R1), VII (R2) and VIII (R1). Peak R1 was found to contain four forms of enzyme (Fig. 4a). The most basic form (pI 9.72) seemed to be a homodimer of the Y1Y1 subunits which could not be observed in the isoelectric focusing pattern of the DEAE-flow-through fraction (Fig. 2). The previously characterized R3a (peak II) and R3b (peak III) are a heterodimer of the Y1Y3 subunits (pI 10.35) and a homodimer of the Y3Y3 subunits (pI 9.95), respectively. The pIs of these three enzymes were in the order Y1Y3 > Y3Y3 > Y1Y1. However, it has been experimentally shown that the pIs of homodimers and heterodimers are in the order YaYa > YaYc > YcYc in the case of rat hepatic enzymes,^{1,11)} and B1B1 > B1B2 > B2B2 in the case of human hepatic enzymes.¹²⁾ Therefore, the Y1 subunit involved in the activity peak with pI 9.72 may be different from the Y1 subunit in R3a (peak II). The other three forms in peak R1 were all heterodimers composed of Y2 subunit and a new type of subunit (Y4). Similarly, peaks III and IV were both homodimers of the Y3Y3 subunits as shown in Fig. 3. It is not clear whether the three forms of the Y2Y4 subunits and two forms of the Y3Y3 subunits were generated by autoxidation as observed in the case of rat hepatic glutathione S-transferase A¹³⁾ or by *in vivo* deamidation as assumed in the case of the human hepatic enzyme.⁴⁾

A linear pH gradient (pH 10.5—6.5) was used in the present study. However, peak R4 was out of this pH range, *i.e.*, it had a broad pI value of more than 10.5. Resolution of peak R4 was found to be incomplete under these conditions. Moreover, peak R4 contained different amounts of Y2 subunit and Y3 subunit. Thus, several forms of the enzyme may be present in peak R4.

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