Rat liver glutathione *S*-transferases

A study of the structure of the basic YbYb-containing enzymes

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A purification scheme was devised that resulted in the resolution of a number of basic glutathione *S*-transferases from rat liver, three of which contained two subunits of molecular mass 23 500 Da (i.e. Yb monomers). These were identified as transferases D, C and A by their elution positions from CM-cellulose and their specific activities towards a variety of substrates. Hybridization, immunotitration and peptide 'mapping' experiments demonstrated that transferases D, C and A comprise Yb₂Yb₂, Yb₁Yb₂ and Yb₁Yb₁ subunits.

The glutathione *S*-transferases are a group of multifunctional detoxification proteins that help prevent carcinogenesis by a variety of mechanisms (Smith *et al.*, 1977).

The transferases can be divided into two groups: the basic and the neutral/acidic transferases. In the rat the basic enzymes (pI 7.5–10.0) have been widely studied and have been defined by their order of elution from CM-cellulose as transferases D, X (or YaYa), C, B, A and AA (Jakoby & Habig, 1980; Scully & Mantle, 1981; Hayes & Clarkson, 1982). By definition, the neutral/acidic enzymes, transferases E and M, have pI values less than 7.5, but, in contrast with the basic transferases, little is known about these enzymes in the rat (Gillham, 1971; Fjellstedt *et al.*, 1973; Pabst *et al.*, 1973).

The subunits that the basic transferases comprise can be resolved into three classes by discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. These have been designated Ya (molecular mass 22 000 Da), Yb (molecular mass 23 500 Da) and Yc (molecular mass 25 000 Da) (Bass *et al.*, 1977; Hayes, 1980).

Mannervik & Jensson (1982) have proposed that there are two distinct monomers of *M_r* 23 500 (Yb and Yb') and that transferases A and C represent the YbYb and YbYb' combinations respectively of these subunits. These workers devised a rapid purification scheme for the rat liver transferases, involving the use of *S*-hexylglutathione affinity chromatography followed by chromatofocusing. Six transferases (peaks I–VI) were resolved by

chromatofocusing, and the three peaks (IV–VI) that were eluted at pH 8.85, 8.43 and 8.20 contained polypeptides of molecular mass 23 500 Da. Although peaks IV and V were identified as transferases A and C, the identity of peak VI was not established, but it was referred to as Yb' Yb' protein, since it cross-reacted with antiserum raised against transferase C but not with antiserum raised against transferase A. However, the relationship between the peak IV, V and VI enzymes may not be as simple as that described by Mannervik & Jensson (1982), since peak VI also contained, in addition to subunits of molecular mass 23 500 Da, polypeptides of molecular mass 23 800 Da.

To prevent possible confusion the term Yb has been used in the present study in a generic sense, to refer to all transferase monomers of molecular mass 23 500 Da, and the individual Yb polypeptides have been numbered according to their decreasing isoelectric points. Hence, according to the hypothesis of Mannervik & Jensson (1982), transferases A and C comprise Yb₁Yb₁ and Yb₁Yb₂ monomers respectively.

A number of workers have proposed that the transferases should be redefined according to their subunit compositions. Although a change in the transferase nomenclature is appropriate, because it explains the overlapping substrate specificities of these enzymes, a new terminology cannot replace that devised by Jakoby and his co-workers before the hypothesis that transferase C is a heterodimer is tested and it is clear which transferase comprises Yb₂Yb₂ monomers.

In the present study a purification scheme was

Abbreviation used: GSH, reduced glutathione.

devised for transferase D. Experiments were performed to establish which basic transferases comprise subunits of molecular mass 23 500 Da and to determine their genetic relationships.

Materials and methods

Enzyme assays

Transferase activity was measured as described previously (Fjellstedt *et al.*, 1973; Habig *et al.*, 1974; Hayes & Clarkson, 1982).

Purification of glutathione *S*-transferases

The enzymes were purified by using standard techniques. The DEAE-cellulose and CM-cellulose steps are based on methods described by Habig *et al.* (1974, 1976) and were used to facilitate the identification of the transferases. The hydroxyapatite, chromatofocusing, glutathione affinity chromatography and *S*-hexylglutathione affinity chromatography have been described previously (Simons & Vander Jagt, 1977; Mannervik & Guthenberg, 1981; Hayes & Clarkson, 1982). The DEAE-cellulose and CM-cellulose steps employed by Habig *et al.* (1974, 1976) were modified to optimize resolution and are described below.

(a) *Separation of basic transferases from neutral/acidic transferases.* About 165 g of frozen (−70°C) liver, obtained from 250 g male Wistar rats, was allowed to thaw partially at 20°C before being blended in 500 ml of ice-cold distilled water. The extract was centrifuged (at 10 000 *g* for 80 min at 4°C) and the supernatant filtered through a plug of glass-wool. The resulting solution was applied to DEAE-cellulose equilibrated and eluted (35 ml/h) with 10 mM-Tris/HCl buffer, pH 8.1 (buffer A). Two columns (3.2 cm × 87.0 cm and 3.2 cm × 58 cm) in series were used. A 0–100 mM-NaCl gradient, in buffer A, was employed, with the use of a 250 ml mixing reservoir, to elute the adsorbed neutral/acidic transferases.

(b) *Resolution of basic transferases.* The transferase activity that was not retarded by the DEAE-cellulose was concentrated by (NH₄)₂SO₄ precipitation and dialysed against three changes, each of 2 litres, of 10 mM-sodium phosphate buffer, pH 6.7 (buffer B). Material that was precipitated during dialysis was removed by centrifugation (at 10 000 *g* for 30 min at 4°C). The supernatant was eluted (25 ml/h, 4°C) with buffer B from a 3.2 cm × 76.0 cm column of CM-cellulose. The column was developed with a 0–105 mM-NaCl gradient in buffer B with the use of a mixing reservoir of 325 ml.

(c) *Purification of transferases X and B.* The fractions from CM-cellulose containing transferase X and transferase B were subjected to a second CM-cellulose step in which the samples and columns were equilibrated with buffer B containing either

2 mM-2-mercaptoethanol or 2 mM-dithiothreitol. Chromatography was performed in columns (1.6 cm × 40.0 cm) that were developed with a 0–55 mM-NaCl gradient in buffer B that also contained 2 mM-2-mercaptoethanol or 2 mM-dithiothreitol.

Immunoprecipitation

Antiserum against transferase C was a gift from Dr. W. B. Jakoby, Section on Enzymes and Cellular Biochemistry, National Institutes of Health, Bethesda, MD, U.S.A. The method used has been described previously (Hayes & Clarkson, 1982).

Hybridization studies

These were performed with the use of 7 M-guanidinium chloride (Kitahara & Soto, 1981) or 8.5 M-urea (Hayes *et al.*, 1981).

Peptide 'mapping'

Tryptic peptide 'maps' were constructed by using the methods described by Ambler (1963). After oxidation with performic acid (Hirs, 1967), the transferases were digested with trypsin (diphenyl-carbamoyl chloride-treated) for 18 h at 37°C (transferase/trypsin ratio 30:1, w/w). The soluble acidic peptides were resolved by high-voltage paper electrophoresis at pH 6.5 in the first dimension followed by high-voltage paper electrophoresis at pH 3.5 in the second dimension. The neutral peptides, which migrated as a single spot after high-voltage paper electrophoresis at pH 6.5, were separated by high-voltage paper electrophoresis at pH 3.5 in the first dimension and descending chromatography, with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) as solvent, in the second dimension. The basic peptides were resolved by high-voltage paper electrophoresis at pH 6.5 in the first dimension and descending chromatography (see above) in the second dimension. The nomenclature used to describe the peptides has been used previously (Hayes, 1980). The 'maps' were developed with a 0.2% solution of ninhydrin in acetone containing about 3% (v/v) of collidine/acetic acid (1:2, v/v) solution.

Amino acid compositions

These were determined by Dr. R. P. Ambler, Department of Molecular Biology, University of Edinburgh, Scotland, U.K., with a Beckman-Spinco model 120-C amino acid analyser (Ambler & Brown, 1967).

Results

Evidence that transferase C is a heterodimer

Initial experiments were performed to determine whether the two monomers that transferase C contains are identical or non-identical. Transferase

C was denatured with either 8.5 M-urea or 6.5 M-guanidinium chloride and renatured by extensive dialysis. The recovery of 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity after urea de-

Fig. 1. Analysis of the products obtained from transferase C after reversible denaturation

Portions of transferase C (about 4.5 mg) were reversibly denatured as described in the text. The soluble products were applied to 2.2 cm × 25 cm columns of CM-cellulose equilibrated and eluted (22 ml/h) with 10 mM-phosphate buffer, pH 6.7. Fractions of volume 3.6 ml were collected, and the Na^+ concentrations (■) and transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) (▲) were determined. The elution profiles obtained after treatment with 7 M-guanidinium chloride (a) and 8.5 M-urea (b) are shown.

naturation was 68% and after guanidinium chloride denaturation it was 73%. Analysis of the renatured products by CM-cellulose chromatography showed that three enzyme-containing peaks, Yb_2Yb_2 , Yb_1Yb_2 and Yb_1Yb_1 , were recovered (Fig. 1).

The position of elution of the Yb_2Yb_2 -containing and Yb_1Yb_2 -containing enzymes from CM-cellulose was dependent on the agent used to denature transferase C: the Yb_2Yb_2 and Yb_1Yb_2 species obtained after urea treatment bound to CM-cellulose more tightly than did those obtained after guanidinium chloride denaturation. This phenomenon appears to be mediated by the Yb_2 monomer, since the position of elution of the Yb_1Yb_1 -containing enzyme from CM-cellulose was constant.

The Yb_1Yb_2 protein and Yb_1Yb_1 protein were provisionally identified as transferases C and A respectively, since their elution positions correspond to those previously obtained for these two proteins (Hayes & Clarkson, 1982). The identity of Yb_2Yb_2 protein is not known, but its elution position from CM-cellulose suggests that it is either a neutral/acidic transferase or one of the basic peak I or peak II transferases (Hayes & Clarkson, 1982). These enzymes were purified to allow the Yb_2Yb_2 -containing enzyme to be identified.

Purification of neutral/acidic transferases

The transferases were resolved into four peaks of activity by DEAE-cellulose chromatography (Fig. 2). The major peak, which was not retained by the exchanger, represents the basic transferases. The neutral/acidic transferases were resolved into three peaks of activity, which were eluted at Na^+

Fig. 2. Elution of rat liver extract from DEAE-cellulose

The soluble extract from 165 g of frozen liver, from male 250 g Wistar rats, was prepared and applied to DEAE-cellulose (bed volume approx. 1175 ml) equilibrated with 10 mM-Tris/HCl buffer, pH 8.1, as described in the text. The flow rate was 36 ml/h, and the fraction volume was 9 ml. The Na^+ concentrations (■) were determined by flame photometry, and transferase activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) (▲) and 1,2-dichloro-4-nitrobenzene (DCNB) (●).

concentrations of 33, 50 and 64 mM; these were designated N1, N2 and N3 respectively. Peak N1 exhibited high activity for 1,2-epoxy-3-(*p*-nitrophenoxy)propane [it possessed a 1-chloro-2,4-dinitrobenzene-GSH-/1,2-epoxy-3-(*p*-nitrophenoxy)propane-GSH-conjugating ratio of about 10:1], demonstrating that transferase E was eluted in this fraction.

Resolution of basic transferases

These were separated by CM-cellulose chromatography into six zones of activity (Fig. 3). Zone I contains transferase D and two minor transferases, which were resolved by chromatofocusing (Fig. 4). Zone II is heterogeneous, and although it contains transferase X it was eluted as two partially resolved

peaks. Fractions 147–160, 161–172 and 173–179 were separately combined, and after further CM-cellulose chromatography three peaks of activity, IIa, IIb and IIc, were resolved, which were eluted at Na⁺ concentrations of 28, 35 and 39 mM respectively. The activity in zone III was attributed to transferase C because of its ability to catalyse the conjugation of 1,2-dichloro-4-nitrobenzene with GSH. Zone IV contains transferase B, and was resolved into two components. These were separately combined, and after a further CM-cellulose step two peaks of activity were resolved, which were eluted at Na⁺ concentrations of 54 mM (IVa) and 60 mM (IVb). The enzyme that was eluted in zone V was identified as transferase A as it possesses both 1,2-dichloro-4-nitrobenzene-GSH-

Fig. 3. Elution of basic transferases from CM-cellulose

The material that was eluted from DEAE-cellulose in the 'flow-through' fractions (575 ml–1170 ml, about 2.3 g of protein) was concentrated by (NH₄)₂SO₄ precipitation and applied to a column (3.2 cm × 76.0 cm) of CM-cellulose equilibrated with 10 mM-phosphate buffer, pH 6.7. Fractions of volume 4.3 ml were collected, and the Na⁺ concentrations (■) were determined. Transferase activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) (▲) and 1,2-dichloro-4-nitrobenzene (DCNB) (●). The activity that was eluted was divided into zones I–VI, which are indicated by arrows, on the basis of previous work (Hayes & Clarkson, 1982). The activity that was eluted in zones II and IV was subdivided into II_α, II_β, II_γ, IV_α and IV_β as shown by the solid bars (see the text).

Table 1. Substrate specificities of glutathione S-transferases

Substrate	Transferase ...	Sp. activity (μmol of product formed/min per mg of transferase)			
		A	C	Id	E*
1-Chloro-2,4-dinitrobenzene†		186	134	91	0.01
1,2-Dichloro-4-nitrobenzene‡		10.1	4.2	1.0	0
1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)propane‡		0.39	0.59	0.69	6.7
<i>trans</i> -4-Phenylbut-3-en-2-one‡		0.11	2.49	3.36	0
1-Menaphthyl sulphate‡		0.02	0.02	0.02	—

* Data for transferase E are from Habig *et al.* (1974).

† Activities obtained by using a centrifugal analyser.

‡ Activities obtained by using manual methods.

Fig. 4. *Chromatofocusing of the basic zone-I transferases*

The basic transferases that were obtained in the zone-I eluate from CM-cellulose were purified by GSH affinity chromatography. The resulting material (30 ml, about 17 mg of protein) was dialysed against 2 litres of 25 mM-ethanolamine/acetate buffer, pH 9.5, and applied to a 1.6 cm × 35.0 cm column of chromatofocusing gel PB 94. This column was eluted (20 ml/h) with polybuffer 96 adjusted to pH 6.5, and fractions of volume 5 ml collected. The pH (□), absorbance at 280 nm (●) and transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) (▲) in the eluate were determined. No transferase activity was detected in the fractions eluted between pH 7.7 and pH 6.5 or in the fractions obtained after elution with 2 M-NaCl.

conjugating and 1-chloro-2,4-dinitrobenzene–GSH-conjugating activity. Zone VI contains transferase AA.

Identification of the Yb₂Yb₂-containing transferase

The purification scheme used in the present study resulted in the recovery of 11 forms of basic transferase activity.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that the transferases purified from zone Id, III and V comprised two subunits of molecular mass 23 500 Da.

Table 1 shows the specific activities of these enzymes for a variety of substrates that have been previously used to identify individual transferases. These activities are consistent with the hypothesis that the enzymes from zones Id, III and V represent the Yb₂Yb₂, Yb₁Yb₂ and Yb₁Yb₁ subunit combinations. These results also suggest that the zone Id enzyme may be identified as transferase D.

The activities of transferases Id (D), C and A were precipitated in parallel by antiserum raised against transferase C, indicating the presence of common antigenic determinants. Transferases from zones II, IV and VI were not precipitated by this antiserum.

The recovery of amino acids from transferases A, C and Id (D) after 18 h hydrolysis (Table 2) also supports the hypothesis that transferase C is a hybrid of transferases A and D.

Table 2. *Amino acid compositions of transferases A, C and Id*

Samples (about 0.5 mg of protein) of transferases A, C and Id were hydrolysed in 6 M-HCl for 18 h as described in the text. The compositions were not corrected for the increased or decreased recoveries of serine, threonine, valine and isoleucine.

Amino acid	Transferase ...	Amino acid composition (μmol recovered from 18 h hydrolysate)		
		A	C	Id
Lys		0.144	0.157	0.168
His		0.034	0.035	0.036
Arg		0.096	0.093	0.089
Asx		0.170	0.168	0.166
Thr		0.047	0.047	0.046
Ser		0.079	0.076	0.070
Glx		0.170	0.161	0.158
Pro		0.074	0.080	0.079
Gly		0.070	0.076	0.085
Ala		0.080	0.073	0.075
Val		0.032	0.038	0.044
Met		0.054	0.051	0.049
Ile		0.079	0.076	0.067
Leu		0.176	0.178	0.176
Tyr		0.093	0.087	0.085
Phe		0.078	0.083	0.088

Hybridization of transferases Id (D) and A

Fig. 5 demonstrates that, after the reversible denaturation of mixtures of transferases Id (D)

Fig. 5. Analysis of the products obtained from transferases Id and A after reversible denaturation. Portions of transferases Id and A (about 2.3 mg of each) were reversibly denatured with 8.5 M-urea as described in the text. The soluble products were applied to 2.2 cm $\times$ 25 cm columns of CM-cellulose equilibrated and eluted (22 ml/h) with 10 mM-phosphate buffer, pH 6.7. Fractions of volume 3.6 ml were collected, and the Na^+ concentrations (■) and transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) (▲) were determined.

Fig. 6. Two-dimensional 'map' of the acidic peptides from transferases Id, C and A. Portions (3–8 mg of protein) of transferases Id, C and A were digested with trypsin as described in the text. 'Maps' of the acidic peptides were prepared by high-voltage paper electrophoresis (h.v.p.e.) at pH 6.5 in the first dimension and high-voltage paper electrophoresis at pH 3.5 in the second dimension. The origin (O) and the position where the paper was cut after electrophoresis in the first dimension (indicated by an arrow) are shown. The positions of migration of alanine (A), glycine (G), glutamic acid (E), Xylene C FF (X) and the red Pentel marker (RP) are shown. The peptides that appear to be common to all three proteins are represented by the blackened spots.

and A, three peaks of transferase activity can be resolved by CM-cellulose chromatography, one of which (Yb_1Yb_2) is co-eluted with native transferase C.

Peptide 'maps': evidence that transferase C is a hybrid of transferases A and Id (D)

The peptide 'map' of transferase Id (D) was constructed and compared with those obtained from transferases C and A, to determine whether this enzyme could represent the putative Yb_2Yb_2 protein. Although transferases D, C and A were highly

insoluble after performic acid oxidation, digestion with trypsin (for 18 h at 37°C) resulted in the dissolution of these enzymes and the construction of reproducible peptide 'maps' (Figs. 6, 7 and 8). Marked differences exist between the maps of the soluble peptides from transferases D and A, but all the peptides from transferases D and A appeared to be represented in the peptide 'maps' of transferase C.

Transferase D yielded a total of 40 tryptic peptides (11 acidic, 13 neutral and 16 basic peptides). Transferase C yielded 59 tryptic peptides (16 acidic, 17 neutral and 26 basic peptides).

Fig. 7. Two-dimensional 'map' of the neutral peptides from transferases Id, C and A

The tryptic digests of transferases Id, C and A were prepared as described. The neutral peptides obtained from the digests after high-voltage paper electrophoresis (h.v.p.e.) at pH 6.5 were subjected to a further high-voltage paper electrophoresis step at pH 3.5 in the first dimension. These peptides were further resolved by descending chromatography with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 16 h in the second dimension. The positions of migration of alanine (A), glycine (G), glutamic acid (E), leucine (L), Xylene C FF (X), valine (V) and proline (P) are shown. The peptides that appear to be common to all three proteins are represented by the blackened spots.

Fig. 8. Two-dimensional 'map' of the basic peptides from transferases Id, C and A

Transferases Id, C and A were digested with trypsin as described in the text. The peptides were separated by high-voltage paper electrophoresis (h.v.p.e.) at pH 6.5. In the second dimension the basic peptides were resolved by descending chromatography with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) as solvent for 16 h. The origin (O) and the position where the paper was cut after electrophoresis (indicated by an arrow) are shown. The positions of migration of the neutral amino acids (N), lysine (K), arginine (R), histidine (H), leucine (L), Xylene C FF (X), valine (V) and proline (P) are indicated. The peptides that appear to be common to all three proteins are represented by the blackened spots.

Transferase A yielded 37 tryptic peptides (seven acidic, 12 neutral and 18 basic peptides). A total of 19 peptides, three acidic (A_1 – A_3), eight neutral (N_1 – N_8) and eight basic (B_1 – B_8) appeared to be

common to all three transferases. It should be noted that transferases D and A yielded respectively four and three more tryptic peptides than would be predicted from their arginine and lysine content. This

may be due to either microheterogeneity or the extended period of digestion that was necessitated by the insolubility of the oxidized transferases. Despite this possible drawback, it was desirable to use oxidized transferases to prepare peptide 'maps', since this method has been used to prepare 'maps' of transferases X and B (Hayes, 1980). The use of oxidized transferases in the present study permits a comparison with previous work.

Discussion

During the present study a purification scheme was developed that has enabled a structural investigation of the basic transferases that contain Yb monomers. In particular, experiments were performed to determine the subunit composition of transferase C, the major transferase in rat liver cytosol.

The two subunits that transferase C comprises both have a molecular mass of 23 500 Da and cannot be distinguished by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Two independent lines of evidence indicate that this enzyme is a heterodimer. Firstly, the two subunits possess charge differences and can be resolved by reversible denaturation, with urea or guanidinium chloride, followed by CM-cellulose chromatography of the renatured products. Secondly, the observation that 59 soluble peptides were resolved from tryptic digests of transferase C indicates that this enzyme is a heterodimer; the amino acid composition predicted that only 35 tryptic peptides would be obtained from transferase C if it were a homodimer. The subunits that this enzyme comprises were called Yb₁ and Yb₂ and had pI values of about 9.0 and 8.0 respectively.

The amino acid compositions, immunotitration, peptide 'mapping' and hybridization experiments suggest that Yb₁ and Yb₂ homodimers also exist in rat liver cytosol; transferase C is a hybrid of transferases A and Id. This conclusion is also supported by the specific activities of these enzymes towards various substrates. The identity of Yb₁Yb₁ protein as transferase A was established by its elution position from CM-cellulose, its substrate specificity, its immunological behaviour and its amino acid composition (Habig *et al.*, 1974; Hayes & Clarkson, 1982). It is, however, less clear what transferase Id has been designated in previous nomenclature. Although I have established that transferase Id (Yb₂Yb₂) represents the Yb'Yb' protein (transferase C₂) described by Mannervik & Jensson (1982), it is less certain what this protein was called by Jakoby and his colleagues (Habig *et al.*, 1974, 1976). The elution of transferase Id from CM-cellulose in zone I indicates that it can be

identified as either transferase D or transferase E. The low activity of transferase Id towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane strongly suggests that this enzyme is transferase D and not transferase E.

The Yb₁ and Yb₂ monomers appear to possess significant sequence homology, since transferases Id (D) and A yield common peptides after tryptic digestion. However, the genes that code for these two subunits are subject to different regulatory control, as only transferase A (Yb₁Yb₁) is induced by phenobarbital (Hayes *et al.*, 1979). Beale *et al.* (1982) have prepared peptide 'maps' of Ya and Yc monomers and have shown that about two-thirds of the total number of peptides are common to both subunits. These workers argued that the Ya and Yc subunits are coded for by two distinct but related genes that have arisen by gene duplication and divergence. Likewise, the Yb₁ and Yb₂ subunits appear to be coded for by two separate genes that have also arisen by the same mechanism. The large differences that exist between the peptide 'maps' of the Yb monomers and the Ya/Yc monomers suggest that, if all the transferases arose from a common ancestral gene, then the genes that code for the Yb monomers are distantly related to those that code for the Ya and Yc subunits.

The transferases have been widely studied because of their ability to bind hydrophobic non-substrate ligands (Ketley *et al.*, 1975; Wolkoff *et al.*, 1978). During the present study cholate-aminohexyl-Sepharose 4B (Pattinson *et al.*, 1980) was used to purify a number of transferases. Pattinson (1981) reported that transferases C, A and AA bound to this affinity matrix. This finding has now been confirmed, and it has also been found that transferase D as well as all the neutral enzymes bind to cholate-aminohexyl-Sepharose 4B. This observation supports earlier work, with the use of equilibrium gel-filtration chromatography, that indicated that the neutral transferases and/or transferase D bound cholic acid (Hayes, 1980).

The hypothesis that transferase C is a heterodimer was originally proposed by Mannervik & Jensson (1982). During the present study this hypothesis was confirmed by the use of both peptide 'mapping' and hybridization techniques, and additional findings were obtained that suggest that transferase C is a hybrid of transferases A and D. Ketterer *et al.* (1983) have independently constructed peptide 'maps' of transferases A, C and D, and have drawn the same conclusions about their genetic relationships. It should be noted that a purification scheme has not been previously reported for transferase D, and the method described in the present paper results in a high yield of this enzyme and will facilitate its further characterization.

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