Human microsomal glutathione *S*-transferase

Its involvement in the conjugation of hexachlorobuta-1,3-diene with glutathione

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A microsomal glutathione *S*-transferase (GST) was purified from human liver. This enzyme was shown to have characteristics similar to those of the rat microsomal GST described by Morgenstern & De Pierre [(1983) *Eur. J. Biochem.* **134**, 591–597]. The specific activity of human microsomal GST towards 1-chloro-2,4-dinitrobenzene or cumene hydroperoxide can be stimulated by treating the enzyme with *N*-ethylmaleimide. This enhancement of activity is accompanied by increased sensitivity to inhibition by haematin and cholic acid. The subunit M_r values of the rat and human enzymes are similar (approx. 17 300), and the proteins are immunologically related. During purification, both human and rat microsomal GST enzymes are the only hepatic proteins obtained from Triton X-100-solubilized microsomal fractions that show activity towards the nephrotoxin hexachlorobuta-1,3-diene. The involvement of microsomal GST in toxification reactions is discussed.

INTRODUCTION

The glutathione *S*-transferases (GST) constitute a multigene family of enzymes that are thought to be pivotal in the protection of cellular macromolecules from reactive products of O_2 and xenobiotic metabolism.

Isoenzymes from rat have been extensively studied, and at least three distinct families of soluble GST exist. These have been referred to as classes Alpha, Mu and Pi, or groups I, II and III (Mannervik *et al.*, 1985; Hayes & Mantle, 1986a). The cytosolic transferases are dimeric and comprise various combinations of subunits that have M_r values ranging from 24 800 to 27 500 (Hayes & Mantle, 1986b).

In addition to the cytosolic enzymes, GST activity has been studied in subcellular membrane fractions. A microsomal GST has been described from rat and mouse liver (Morgenstern *et al.*, 1982; Andersson *et al.*, 1988), but, to date, other forms of microsomal GST have not been reported (for a review see Morgenstern & De Pierre, 1985). The microsomal GST is distinct, in that it is thought to be a trimeric protein with three identical subunits of M_r 17 237 (Morgenstern *et al.*, 1985; Boyer *et al.*, 1986). Furthermore, unlike cytosolic GST, the specific activity of purified rat microsomal GST towards 1-chloro-2,4-dinitrobenzene (CDNB) and cumene hydroperoxide can be stimulated by treatment with thiol-blocking reagents (Morgenstern & De Pierre, 1983; Morgenstern *et al.*, 1987).

GST isoenzymes analogous to members of the three groups of rat cytosolic GST are expressed in human tissue, and have been isolated and characterized (Hayes *et al.*, 1983, 1987a,b,c; Warholm *et al.*, 1983; Dao *et al.*, 1984; Stockman *et al.*, 1985, 1987). However, microsomal GST has not been purified hitherto from human tissue and its existence has been in some doubt. Morgenstern *et al.* (1984) showed that GST activity in the microsomal

fractions (referred to below simply as microsomes) of all mammalian livers examined, except human, increased after incubation with *N*-ethylmaleimide (NEM).

Conservation of the biochemical properties of microsomal GST in mammalian liver is particularly important, since the GST associated with the microsomal fraction in rat liver has been implicated in mediating the toxicity of certain exogenous compounds, notably the potent nephrotoxin hexachlorobuta-1,3-diene (HCBD) (Wolf *et al.*, 1984). It was previously reported that rat hepatic microsomes display significantly higher GST activity towards HCBD as a substrate than do liver cytosols (Wolf *et al.*, 1984), but the identity of the GST involved was not established. Microsomal GST purified from rat liver has, however, been reported to have some activity (5 nmol/min per mg of protein) with this substrate (Morgenstern & De Pierre, 1983).

In the present paper we describe the purification and characterization of a human hepatic microsomal GST that is closely related to the rat microsomal GST described by Morgenstern *et al.* (1982). Evidence is presented that in both species this isoenzyme is involved in the microsomal conjugation of HCBD with GSH.

EXPERIMENTAL

Animals

Adult male Sprague–Dawley rats (200–250 g) were purchased from Bantin and Kingman, Hull, U.K. Animals were fasted overnight before being killed, to lower the glycogen content of the liver.

Human tissue

Human liver samples were obtained from patients where traumatic injury was the cause of death. Livers were obtained within 2 h of death and were frozen

Abbreviations used: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; HCBD, hexachlorobuta-1,3-diene; NEM, *N*-ethylmaleimide.

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immediately in liquid N₂. The liver samples were stored at -80 °C until use. The purification of microsomal GST was undertaken from livers from patients 7 and 8 described by Hussey *et al.* (1986).

Chemicals

Radiolabelled [¹⁴C]HCBd was synthesized by Physics and Radioisotopes Services, Imperial Chemical Industries, Billingham, U.K., and was generously given by Dr. E. A. Lock. Androst-5-ene-3,17-dione was a gift from Dr. P. K. Stockman. Cocktail T Scintiran scintillation fluid was purchased from BDH Chemicals, Poole, Dorset, U.K. All other chemicals were of analytical grade and readily available commercially.

Buffers

The pH values of buffers were determined at 4 °C: buffer A, 150 mM-Tris/HCl buffer, pH 8.0; buffer B, 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-GSH, 0.1 mM-EDTA, 1 % (v/v) Triton X-100 and 20 % (v/v) glycerol.

Analytical

Protein determination. Protein concentrations were determined by the method of Peterson (1977).

Enzyme assays. All enzyme assays were carried out at 37 °C. Assays for GST activity with CDNB as substrate were performed as described by Morgenstern *et al.* (1980). Enzymic activity with ethacrynic acid, androst-5-ene-3,17-dione and *p*-nitrobenzyl chloride was measured at 37 °C as described by Habig & Jakoby (1981). Glutathione peroxidase activity towards cumene hydroperoxide was determined by using the coupled assay system described by Reddy *et al.* (1981).

The assay for microsomal GST with HCBd as substrate was carried out by a modification of the method of Wolf *et al.* (1984). The assay system contained 5 mM-GSH, 1 mM-HCBd and 0.2 M-potassium phosphate buffer, pH 7.4, and had a final volume of 200 µl that contained 0.2 µCi of [¹⁴C]HCBd. The reaction was started by the addition of HCBd, as a solution in 10 µl of ethanol, and incubation was carried out for 1 h, in a shaking water bath, at 37 °C. Samples were placed on ice to terminate the reaction, and 10 µl portions were applied to t.l.c. plates [polyester sheets with silica-gel adsorbent (Eastman Kodak Co., Rochester, NY, U.S.A.)]. Chromatography was carried out with an Eastman chromatogram chamber plate set, with butan-1-ol/acetic acid/water (12:3:5, by vol.) as the running solvent. The t.l.c. plates were cut into 1 cm² strips and mixed with 1 ml of water in scintillation vials, followed by the addition of 5 ml of scintillation fluid. Radioactivity was determined with a Packard model 3255 liquid-scintillation spectrometer. Conjugated HCBd, in this t.l.c. system, had an *R_F* value of 0.42; this compares with a value of 0.33 reported by Wolf *et al.* (1984).

***K_m* determination.** *K_m* values for GSH and CDNB were determined by constructing Hanes plots (Dixon & Webb, 1979) and calculating the values by linear-regression analysis, by using the computational method described by Wilkinson (1961).

Inhibition of GST activity. Haematin or cholic acid was added to enzyme assay mixtures, in increasing

concentrations, and the conjugation rate of GSH with CDNB was measured. The *I*₅₀ value, which is the concentration of compound at which 50 % inhibition of enzyme activity is achieved under standard assay conditions (i.e. 5 mM-GSH, 1 mM-CDNB), was determined for each non-substrate ligand (Yalçin *et al.*, 1983).

Electrophoresis and immunoblotting. SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) and Western blotting (Towbin *et al.*, 1979) were performed as described previously (Hayes & Mantle, 1986a). SDS/polyacrylamide gels contained 15 % (w/v) polyacrylamide that incorporated 0.4 % (w/v) *NN'*-methylene-bisacrylamide.

Preparation of hepatic microsomes

These were prepared from human and rat liver by essentially the same method; the only significant difference was that rat microsomes were prepared from unfrozen liver whereas human hepatic microsomes were from tissue that had been frozen. Thawed or fresh liver tissue was minced and then homogenized in 3 vol. of ice-cold buffer A. The homogenates were centrifuged at 10000 *g* for 20 min at 4 °C. Sediments were discarded and the supernatants were centrifuged at 100000 *g* for 60 min at 4 °C; the 100000 *g* sediments were retained and further processed. Microsomes were separated from glycogen as described by McLellan & Hayes (1987), and were washed by resuspending the protein sediment in a volume of buffer A corresponding to approximately half the volume of 10000 *g* supernatant from which the microsomes were prepared (Morgenstern *et al.*, 1982). The particulate matter was harvested by centrifugation (100000 *g* for 60 min at 4 °C) and the washing procedure was repeated.

Treatment of microsomes before purification of GST

Rat hepatic microsomes were treated with NEM, and the GST activity was solubilized with Triton X-100 at 4 °C, as described by Morgenstern *et al.* (1982). The microsomal fraction from human liver was also solubilized with Triton X-100 by a similar method, but was given no prior treatment with NEM.

Washed human microsomes (from approx. 55 g of liver) were resuspended in 14 ml of 250 mM-sucrose, and 9 ml of 10 mM-potassium phosphate buffer, pH 7.0, was added. Solubilization was carried out by the dropwise addition of 4 ml of 10 % (v/v) Triton X-100, over the course of 5 min, with gentle stirring of the microsomal fraction. Glycerol (8 ml) and 300 µl of 1 M-potassium phosphate buffer, pH 7.0, were then added and mixing was continued for a further 20 min.

Purification of microsomal GST

Rat and human hepatic microsomal GST enzymes were purified by a similar method to that described by Morgenstern *et al.* (1982). The solubilized protein (400–500 mg) was applied to hydroxyapatite columns (2.2 cm × 28 cm) that had been equilibrated with buffer B. Columns were developed with a gradient of 10–300 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-GSH, 0.1 mM-EDTA, 1 % (v/v) Triton X-100 and 20 % (v/v) glycerol throughout. The eluate was monitored for GST activity with both CDNB and HCBd as substrates. The major peak of GST activity

from hydroxyapatite was de-salted by gel filtration on Sephadex G-25 columns (4.4 cm $\times$ 80 cm) that had been equilibrated with buffer B.

Final purification of microsomal GST was achieved by cation-exchange chromatography on CM-Sepharose columns (2.2 cm $\times$ 11 cm) equilibrated with buffer B. The GST-containing microsomal protein was applied to CM-Sepharose, which was then developed with a gradient of 0–200 mM-KCl in buffer B. The eluate was monitored for GST activity with both CDNB and HCBD.

Preparation of antisera

Antisera were raised against purified rat microsomal GST, in New Zealand White rabbits, as described by Hayes & Mantle (1986a).

RESULTS

Comparison of microsomal GST in individual human livers

Microsomal GST was purified from rat liver and antiserum was raised against the purified protein. These antibodies were used to analyse human microsomal preparations from individual human livers, by Western blotting. Every liver examined (a total of seven were studied) was found to contain a polypeptide (M_r 17300) that was immunologically related to rat microsomal GST.

Treatment of two separate human microsomal preparations with NEM did not result in activation of GST activity with CDNB. Instead, inhibition of activity was seen in both cases; activity was decreased from 0.09 μ mol/min per mg to 0.07 μ mol/min per mg.

Purification of human microsomal GST

In view of the inhibitory effects of NEM on human hepatic microsomal GST activity, human microsomal GST was purified without prior treatment of microsomes with NEM.

Microsomal GST was purified from two human livers, and both microsomal preparations yielded closely similar elution profiles. Hydroxyapatite chromatography

resulted in the elution of one major peak of activity, at 110–130 mM-potassium phosphate buffer, pH 7.0. Activities with CDNB and HCBD were coincident (Fig. 1). The pool of activity was de-salted on Sephadex G-25 and final purification was achieved by chromatography on CM-Sepharose. This resulted in the resolution of three peaks of GST activity towards CDNB, which were named A, B and C respectively. Only peak C, however, was found to have significant activity with HCBD as substrate (Fig. 2).

Identification of GST isolated from human liver microsomes

The identities of the GST in the three peaks from CM-Sepharose were established by SDS/polyacrylamide-gel electrophoresis analysis and immunoblotting. Peaks A and B were both found to contain proteins with a subunit M_r of 25900; this corresponds to the M_r of the cytosolic GST isolated from the liver of the same individual (results not shown). The polyacrylamide-gel analysis of peak C (Fig. 3) showed that this GST comprised subunits with an M_r analogous to that of rat microsomal GST (apparent M_r 17300).

Peaks A, B and C were analysed by immunoblotting, with antisera raised against the human 'basic' cytosolic GST B_1B_1 and GST B_2B_2 and rat microsomal GST (Fig. 4). The GST in peaks A and B both cross-reacted with anti-(GST B_1B_1) IgG and anti-(GST B_2B_2) IgG, but not with anti-(rat microsomal GST) IgG, whereas the protein in peak C cross-reacted with antiserum raised against rat microsomal GST, but showed no immunological identity with either 'cytosolic-type' GST. The protein in peak C was therefore positively identified as human microsomal GST, whereas peaks A and B are likely to contain cytosolic GST, which partition into the microsomal fraction.

During purification of microsomal GST from another human liver known to contain GST μ , a transferase that showed immunological cross-reactivity with GST μ (subunit M_r 26700) was also found to be associated with the microsomal fraction. This GST was eluted from CM-Sepharose in a position similar to that of the human 'basic' cytosolic GST.

Fig. 1. Elution of human liver microsomal GST from hydroxyapatite

Hydroxyapatite chromatography of solubilized human microsomes was performed as described in the text. The flow rate was 13.8 ml/h, and 5 ml fractions were collected. GST activities with CDNB ($\blacktriangle$) and HCBD ($\square$) were measured, and potassium phosphate concentrations ($\blacksquare$) were monitored. Fractions were pooled as indicated by the horizontal bar.

Fig. 2. Purification of human microsomal GST on CM-Sepharose

Microsomal GST was purified by cation-exchange chromatography as described in the text. The flow rate was 13.8 ml/h, and 2.2 ml fractions were collected. GST activities with CDNB ($\blacktriangle$) and HCBP ($\square$) were measured, and concentrations of K^+ ($\blacksquare$) were monitored. The fractions that were pooled are indicated by the horizontal bars; these were designated A, B and C by their order of elution.

Catalytic properties of microsomal GST

Activity of the microsomal GST towards a variety of substrates, and the effects of incubating the enzyme with 2 mM-NEM, were determined (Table 1). As with rat microsomal GST (Morgenstern & De Pierre, 1983), the activity of the purified human microsomal GST with CDNB and cumene hydroperoxide is stimulated by treatment with NEM. The extent of activation and the basal specific activity showed small variations between separate microsomal GST preparations from the same liver, and from the second liver (specimens 7 and 8, described by Hussey *et al.*, 1986), and, although up to 5-fold activation with CDNB as a substrate was achieved on one occasion, 2–3-fold activation was generally found. Glutathione peroxidase activity towards cumene hydroperoxide is stimulated by NEM to a greater extent (7.5-fold activation) than is GST activity for CDNB. These values compare with 15-fold stimulation of GST activity with CDNB and 10-fold stimulation of glutathione peroxidase activity reported for purified rat microsomal GST (Morgenstern & De Pierre, 1983). Enhancement of specific activity by NEM was not observed for any of the other substrates examined. The addition of NEM alone to the assay mixture was found to stimulate the non-catalytic isomerization rate of androst-5-ene-3,17-dione. Addition of the enzyme previously incubated with NEM to the reaction mixture did not further increase the isomerization rate.

The K_m values for GSH and CDNB were determined as previously described. Although slight deviations from linearity were observed, the estimated K_m values of the activated and unactivated microsomal GST were found to be similar (Table 2).

HCBP as a substrate for microsomal GST

During purification of human microsomal GST (Figs. 1 and 2) activity of HCBP was found to be co-eluted with the protein corresponding to the microsomal GST. Column eluates obtained during purification of rat

microsomal GST were also monitored for activity with HCBP. Although, for both chromatography steps, only a single GST activity peak with CDNB as substrate was obtained, in each case the activity with HCBP was co-eluted with activity for CDNB (results not shown). The purified rat microsomal GST was found to have a specific activity towards HCBP of $0.01 \mu\text{mol/min per mg}$; this compares with a value of $0.006 \mu\text{mol/min per mg}$ for the equivalent human GST.

Effects of non-substrate ligands on microsomal GST

Two non-substrate ligands, cholic acid and haematin, were investigated as potential inhibitors of human microsomal GST activity; I_{50} values were determined for both compounds. The microsomal enzyme was studied

Table 1. Substrate specificity of human microsomal GST

For experimental details see the text. Abbreviation: N.D., not determined.

Substrate	Activity of microsomal GST ($\mu\text{mol/min per mg}$)		Activation (fold)
	Control	NEM-treated	
CDNB	1.88	4.45	2.5
Cumene hydroperoxide	0.12	0.92	7.5
Ethacrynic acid	< 0.01	< 0.01	0
Androst-5-ene-3,17-dione	0.03	N.D.*	–
<i>p</i> -Nitrobenzyl chloride	0.57	0.59	0
HCBP	0.006	N.D.	–

* Rate of isomerization was stimulated by NEM when no GST was present.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of human and rat microsomal GST enzymes

Approx. 2 μ g portions of purified microsomal GST from human and rat liver were analysed by SDS/polyacrylamide-gel electrophoresis in a 15% (w/v) polyacrylamide resolving gel (Laemmli, 1970). The gel was loaded as follows: lane 1, human microsomal GST (peak C from CM-Sepharose); lane 2, rat microsomal GST; lane 3, M_r reference proteins: ovotransferrin (M_r 76000–78000), albumin (M_r 66250), ovalbumin (M_r 45000), chymotrypsinogen A (M_r 27500), myoglobin (M_r 17200) and cytochrome *c* (M_r 12300); lane 4, M_r reference proteins: carbonic anhydrase (M_r 30000) and trypsin inhibitor (M_r 24000).

in both the NEM-activated and the unactivated state. For both cholic acid and haematin the activated form of microsomal GST was found to be more sensitive to inhibition than was the unactivated form. In its activated form microsomal GST is particularly sensitive to low concentrations of haematin, the I_{50} value being 1 μ M; the I_{50} value for the unactivated enzyme is 15 μ M. The activated sample has a lower specific activity at 5–10 μ M-haematin than has the unactivated sample.

A similar trend was observed with inhibition by cholic acid, with the NEM-activated sample being more sensitive to inhibition than the unactivated sample. However, concentrations of cholic acid of up to 5 mM were found to stimulate the activity of the unactivated enzyme to a maximum level of 2.5 μ mol/min per mg; concentrations greater than 5 mM had an inhibitory effect. No stimulation of activity was observed for the activated enzyme. Cholic acid is not inhibitory in a micromolar concentration range for either enzyme state. The I_{50} values for the two compounds are summarized in Table 2.

Fig. 4. Immunoblot analysis of GST isolated from human liver microsomes

The peaks of microsomal GST activity, isolated from CM-Sepharose, were analysed by Western blotting (Towbin *et al.*, 1979). Proteins were separated by SDS/polyacrylamide-gel electrophoresis in 15% (w/v) resolving gels before transfer to nitrocellulose paper. The gels were loaded as follows: lane 1, human cytosolic GST, isolated from the same liver as the microsomal GST; lane 2, pool A from CM-Sepharose; lane 3, pool B from CM-Sepharose; lane 4, pool C from CM-Sepharose; lane 5, purified rat microsomal GST. Panels (a), (b) and (c) show immunoblots of the gel with antisera raised against the human basic cytosolic GST B_2B_2 or B_1B_1 (Stockman *et al.*, 1987) and rat microsomal GST respectively.

DISCUSSION

Identification of human microsomal GST

Doubt has been expressed about the existence of a microsomal GST in human liver (Morgenstern *et al.*, 1984). The present study describes the purification of microsomal GST from both rat and human liver, by hydroxyapatite and cation-exchange chromatography. The purification scheme resulted in the isolation of a protein with an apparent subunit M_r of 17300 from both species. This is the first report of the purification of GST from human liver microsomes.

Table 2. Kinetic parameters of human microsomal GST

Microsomal GST	Kinetic parameters				
	K_m (mM)		$V_{max.}$ (μ mol/min per mg)	I_{50} value (μ M)	
	GSH	CDNB		Haematin	Cholic acid
Unactivated	2.95	0.046	3.2	15	12.0×10^3
Activated	3.50	0.053	8.8	1	2.8×10^3

Activation of microsomal GST

GST activity of CDBN in unresolved human microsomal preparations was not increased by NEM treatment. However, levels of enhancement of activity for the purified human enzyme (2.5-fold activation) were significantly lower than for the rat homologue, for which a 15-fold increase in activity could be achieved by NEM treatment (Morgenstern & De Pierre, 1983). It is possible that any stimulation of activity of the microsomal GST in human microsomes by NEM is masked by the effects on cytosolic GST isoenzymes that are associated with the microsomes; Strange *et al.* (1984) have reported that NEM is inhibitory to certain human cytosolic GST isoenzymes. It must be noted also that prolonged freezing can lessen the extent of NEM activation (Morgenstern *et al.*, 1980), and the human livers used in the present study had been frozen for several months.

Non-substrate ligands

It has been shown that, for both rat and mouse microsomal GST enzymes, certain inhibitory compounds have higher I_{50} values for the unactivated enzyme than for the activated form (Morgenstern & De Pierre, 1985; Andersson *et al.*, 1988). NEM-activated microsomal GST from rat is strongly inhibited by low concentrations of haematin and bromosulphophthalein, and the I_{50} values are different for activated and unactivated forms. Table 2 shows that a similar situation exists for human microsomal GST with respect to inhibition by haematin; the I_{50} values for haematin are 1 μ M and 15 μ M respectively, for the NEM-activated and unactivated human enzyme. The I_{50} value of 1 μ M for the human NEM-activated microsomal GST is lower than that described for the human cytosolic GST B₂B₂ (I_{50} 40 μ M) and GST π (I_{50} 5 μ M), and similar to the values for GST B₁B₁ (I_{50} 1.5 μ M) and GST μ (I_{50} 1.0 μ M) (Tahir *et al.*, 1985; Stockman *et al.*, 1987). A similar effect to that of haematin was observed with cholic acid inhibition, where the NEM-activated enzyme was more sensitive to inhibition by cholic acid than was the unactivated form. Concentrations of cholic acid up to 5 mM were found to stimulate the activity of the untreated GST (approx. 1.5-fold), but no increase in activity of the NEM-treated enzyme was observed. This may represent an additional control mechanism for microsomal GST activity *in vivo*: levels of activity could be enhanced by increased intracellular concentrations of bile acids (e.g. during cholestasis).

Microsomal GST and toxification reactions

It is likely that microsomal GST has multiple cellular functions, and, in addition to a putative role in

detoxification, there is now evidence to suggest that this microsomal GST is involved in mediating the toxicity of certain compounds, which include the nephrotoxin HCBD. It was reported previously that hepatic microsomal preparations from rat had a significantly higher specific activity towards HCBD than did rat liver cytosols (Wolf *et al.*, 1984). The identity of the HCBD-conjugating enzyme was unknown, but the present work demonstrates that the microsomal GST, purified from both human and rat liver, has HCBD-conjugating activity. This enzyme was the only GST detected in microsomes with activity for HCBD. It would appear that the GST described in the present study plays a significant role in the nephrotoxicity of HCBD, but, in view of the inhibitory effect of Triton X-100 (Oesch & Wolf, 1989), the presence of other GST enzymes with this activity cannot be precluded.

In addition to the postulated role of microsomal GST in the toxicity of HCBD, this enzyme may therefore also be indirectly responsible for the nephrotoxicity of other haloalkenes. Both chlorotrifluoroethylene and tetrafluoroethylene may be conjugated with GSH, and it is thought that, like HCBD, the mechanism for the nephrotoxicity of these compounds is also mediated by the action of cysteine-conjugate β -lyase on the cysteine conjugate formed during mercapturic acid biosynthesis. The specific activity of hepatic microsomes from rat, for both substrates, is higher than that of cytosol (Dohn & Anders, 1982; Odum & Green, 1984), but the identity of the enzyme(s) responsible for conjugation remains to be established.

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