Preparation and Assay of Glutathione Thiol Esters. Survey of Human Liver Glutathione Thiol Esterases†

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ABSTRACT: Hydrolytic activities toward glutathione thiol esters have been partially purified from human liver. The activities have been characterized by using nine newly synthesized glutathione thiol esters. These have been purified by column chromatographic procedures and standardized by measuring formed GSH after enzymic hydrolysis or decomposition by hydroxylamine. The results suggest that in addition to *S*-2-hydroxyacylglutathione hydrolase (glyoxalase II, EC 3.1.2.6) specific thiol esterases exist in human liver at least for *S*-formyl- and *S*-succinylglutathione. The new thiol esterases have been chromatographically separated from glyoxalase II. Denaturation experiments at low pH, high pH, and high temperature indicate that part of the hydrolysis of *S*-acetyl- and *S*-propionylglutathione is separate from glyoxalase II but the specific thiol esterases appear to be without activity toward *S*-propionylglutathione which suggests the presence of an additional hydrolase. The thiol esterase which specifically catalyzes the hydrolysis of *S*-formylglutathione is not identical with formaldehyde dehydrogenase (EC 1.2.1.1.).

A group of hydrolases with specificity toward thiol esters (EC 3.1.2) has been described from animal tissues. Most of these studies have been made with enzymes specific for thiol esters of CoA[1] (see the references from Anderson and Erwin, 1971). Little is known about enzymes which can catalyze the hydrolysis of thiol esters of glutathione. Three enzymes are mentioned in the list of the Enzyme Commission (1961), but in the last two studies on the subject the conclusion was drawn that only one enzyme, glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6), exists (Wieland *et al.*, 1956; Drummond and Stern, 1961). Racker (1951) separated glyoxalase II from the previous glyoxalase system. The enzyme was purified 20-fold from beef liver. Conflicting results have previously been obtained on the existence of additional thiol ester hydrolases. Thus Kielley and Bradley (1954) found from mouse liver an enzyme specific for *S*-acetyl- and *S*-butyrylglutathione. The activity was separated from glyoxalase II by denaturation of the latter in acid pH. However, Wieland *et al.* (1956) tried without success to reproduce the results of Kielley and Bradley (1954) with beef liver preparations. Strecker *et al.* (1954, 1955) and Sachs and Waelsch (1955, 1957) studied the hydrolysis of several glutathione thiol esters in crude brain and liver preparations. Variation in relative activities with different substrates in different ammonium sulfate fractions suggested the presence of more than one enzyme (Strecker *et al.*, 1954). Decker and Lynen (1955) described a hydrolase specific for *S*-acetoacetylglutathione. However, the studies of Drummond and Stern (1961) well supported those of Wieland *et al.* (1956). This led to the conclusion that the hydrolysis of all glutathione thiol esters studied, including acetoacetyl-SG, was catalyzed only by glyoxalase II.

The results of the present work suggest that at least three chromatographically separable glutathione thiol esterases are found in human liver and some evidence of a fourth one has also been obtained.

Experimental Section

Synthesis and Purification of Substrates. *S*-Lactyl-, *S*-glycolyl-, *S*-glyceryl, and *S*-mandelylglutathione were prepared enzymically (Racker, 1951). Potassium phosphate buffer (0.05 M, pH 6.6), 1.2 equiv of GSH compared to ketoaldehyde, and commercial yeast glyoxalase I preparations (Sigma, Boehringer) were used. Advance of reactions was followed from dilutions with a Beckman DU spectrophotometer at 240 nm. The yield of *S*-lactylglutathione was over 95%. *S*-Mandelyl- and *S*-glycerylglutathione were obtained in 80% yield and *S*-glycolylglutathione in 60% yield. Two column chromatographic procedures were used for purification of the substrates. In the first one the mixture, concentrated to 2 ml *in vacuo*, was applied to a Sephadex G-10 column (2.0 × 50 cm) equilibrated and eluted with water. A rapid separation from enzyme and GSSG was obtained, but GSH and the thiol esters were eluted together. Alternatively, a Dowex 1 ion exchange resin (X-4, 200–400 mesh, column 2.0 × 50 cm) was used. All the four thiol esters behaved differently in Dowex 1. Mandelyl-SG was easily separated from GSH since only the latter was eluted with 5 mM HCl. The thiol ester was then eluted with 90% recovery with 10 mM HCl. Lactyl-SG was purified by Dowex 1 in formate form. A thiol ester entirely free from GSH was recovered with 90% yield (Figure 1). Glycolyl-SG was eluted close to GSH, but owing to good resolution 60% of the peak could be taken free from GSH. However, in contrast to the previous thiol esters glycolyl-SG was unstable in Dowex 1 and about half of it was hydrolyzed to GSH during the run. Glyceryl-SG was eluted slightly before GSH. Some mercaptan-free product was collected from the initial part of the peak for spectral and other special purposes. The thiol esters were then concentrated and freed from formic acid by lyophilization. Lactyl-SG and mandelyl-SG could also be concentrated in a rotary evaporator at 27° without decomposition. The concentrated mandelyl-SG was freed

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[1] Abbreviations used are: CoA, coenzyme A; acyl-CoA, *S*-acylcoenzyme A; GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); acyl-SG, *S*-acylglutathione; Nbs., 5,5′-dithiobis(2-nitrobenzoate); MalNEt, *N*-ethylmaleimide.

from NaCl by Sephadex G-10 gel filtration (load 2 ml, 2 × 50 cm and elution with H_2O).

S-Acetylglutathione was made according to Kielley and Bradley (1954) from GSH and thiolacetic acid with a yield of 80%. The mixture was extracted two or three times with ether at pH 3.0 to remove part of the thiolacetic acid and was, after concentration, further purified by Sephadex G-10 gel filtration (2.0 × 50 cm, load up to 4 ml, eluted with 0.01 M acetate (pH 4.5). The peaks of acetyl-SG and thiolacetic acid were well separated. SH group analysis with Nbs_2 (Ellman, 1959) gave a positive reaction only in the region of the second peak, indicating that the thiol ester was free from glutathione.

S-Propionylglutathione was prepared analogously with thiolpropionic acid as acylating agent. The reaction time was somewhat longer (6–7 hr) in 65% ethanol. Purification was as for acetyl-SG; mere extractions with ether were also efficient. The yield was only 45%, but the amount of remaining GSH was not more than 4–6% of the thiol ester. This impurity could be removed by Dowex 1 formate in which propionyl-SG behaved analogously to lactyl-SG.

S-Acetoacetylglutathione was prepared by acylating GSH with diketene (Lynen *et al.*, 1958) with 95% yield. The product was freed from diketene by storing for several days at −20° before use (Stern and Drummond, 1961).

S-Succinylglutathione. GSH in a minimum amount of water was neutralized to pH 6.6 with NaOH. Succinic anhydride (0.1 M) in ethanol (1.5 equiv) was added and the pH readjusted to 6.6 with NaOH. The reaction was continued for 60–90 min at 0°. The yield was 70–80%. The solution was then stored at 0° for several hours together with a control without GSH until the latter gave a negative reaction in the hydroxamate procedure of Lipmann and Tuttle (1945), indicating that succinic anhydride was destroyed. Succinyl-SG was well separated from GSH by Dowex 1 formate, but the yield was only 35–40%. In addition, GSH-free succinyl-SG appeared to be extremely unstable in neutral pH and 25°, so that enzymic hydrolysis became difficult to measure. Therefore a product only purified by storage was used in this study.

S-Formylglutathione. Equivalents of both formic acid (98–100%) and acetic anhydride were combined and mixed for 60 min at 23° to prepare acetic formic anhydride. Thioglycolic acid (0.2 equiv) was then added and stirring continued for a further 60 min at 23°. Then 100 equiv of water was added to hydrolyze excess anhydride and the mixture was concentrated to dryness at 27° in a rotary evaporator. The yield of acyl thioglycolate was 90–95%, but paper chromatographic analysis of hydroxamates (Sly and Stadtman, 1963) showed that about 20% of the product was acetyl thioglycolate.

To GSH dissolved in a minimum of water 10 equiv of formyl thioglycolate was added. The pH of the solution was adjusted with 6 M NaOH to 5.0 with continuous stirring and cooling. After 60-min incubation at 23° the mixture was cooled to 0°, acidified to pH 2.5–3.0 with 12 M HCl, and extracted at least ten times with 2 volumes of ether to free the solution from formyl thioglycolate. Alternatively, the solution was after only two or three extractions purified by Sephadex G-10 gel filtration (column as before, load up to 5 ml). In this way two other uv-absorbing impurities were removed in addition to traces of *S*-formyl thioglycolate and thioglycolic acid. The yield was only 25% and the rest was present as GSH. Attempts to remove GSH by ion-exchange chromatography failed due to the lability of *S*-formylglutathione. No acetyl-SG was present according to paper chromatography of the hydroxamate, which is in accordance with the conclusion of Stadtman (1952) that the acetyl group is not transferred from thioglycolate to GSH below pH 7.0.

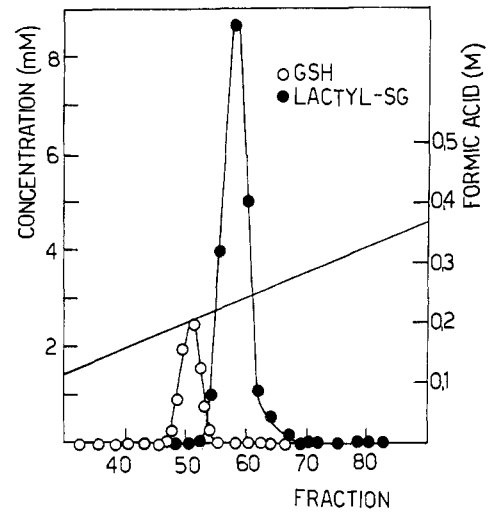

FIGURE 1: Separation of *S*-lactylglutathione from GSH by Dowex 1 column chromatography. The column (2.0 × 50 cm) was eluted with a linear formic acid gradient at 4°. The flow rate was 100 ml/hr and 10-ml fractions were collected. At least 500 μmol of thiol ester were successfully purified by the column.

Standardization of Substrates. The hydroxamate method (Lipmann and Tuttle, 1945; Ibqal and Ottaway, 1970) was used in standardization of acetyl-SG, succinyl-SG, and propionyl-SG with respective acid anhydrides as standards after complete removal of the starting materials. Acetoacetyl thio esters cannot be measured by this method (Stadtman, 1957), and for the other esters a suitable standard was not available. The other substrates were therefore standardized on the assumption that a stoichiometric amount of GSH is formed when the thiol ester bond is broken enzymically or with hydroxylamine. The reactions were followed at 412 nm in the following mixture: 0.1 M Tris-HCl buffer (pH 7.40), thiol ester, 0.15 mM Nbs_2 and either a suitable amount of purified glyoxalase II (Uotila, 1973) or 0.2 ml of freshly neutralized 2 M hydroxylamine in a total volume of 3.0 ml. Decrease of absorbance was measured from a similar mixture without Nbs_2 at 240 nm to obtain the molar absorptivities of the thiol ester bond of the various substrates (Table I). In calculations ϵ_M 13600 (Ellman, 1959) was used for the 5-thio-2-nitrobenzoate ion. Since the p*K* of the colored ion is 5.1 (Ellman, 1958), the value given by Ellman at pH 8.0 practically holds true also at pH 7.4. Also, hydroxylamine in the concentration used was without effect on the constant of Ellman. The reactions were as a rule allowed to go to completion. Also comparison of initial velocities at 412 and 240 nm in enzymic hydrolysis with appropriate blanks was possible, since glyoxalase II is not inhibited by the amount of Nbs_2 used (Uotila, 1973). For acetyl-SG and propionyl-SG the results closely agreed with those obtained by the method of Lipmann and Tuttle (1945). The presence of some GSH could be allowed, but *S*-formylglutathione contained too high an amount of it. This substrate was first treated with 1.6 equiv of *N*-ethylmaleimide compared to GSH in 0.05 M potassium phosphate buffer (pH 6.8) at 0° until the reaction was complete as judged by decrease of absorbance at 300 nm measured from small aliquots (Gregory, 1955). The mixture was then extracted ten times with 2 volumes of ether to remove unbound MalNEt quantitatively. A product initially negative in Nbs_2 assay resulted. This preparation was standardized enzymically as described above. *S*-

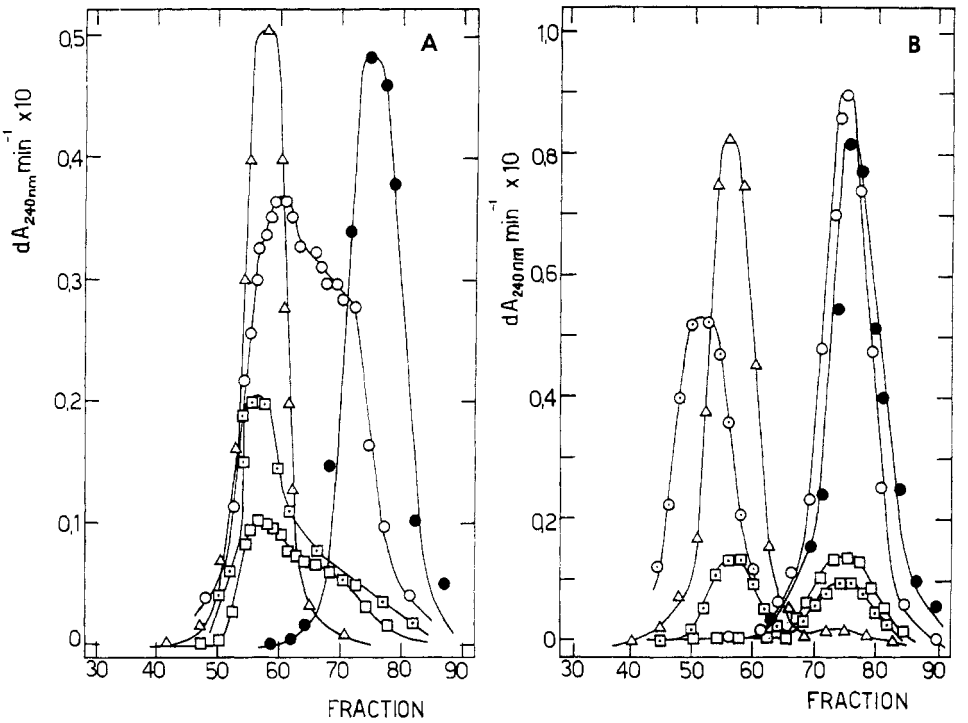

FIGURE 2: Purification of human liver glutathione thiol esterases by Sephadex G-100 gel filtration: (A) eluted with 10 mM buffer, (B) eluted with 210 mM buffer (for details see text). Formaldehyde dehydrogenase (Figure 2B) was assayed by measuring NADH formed from HCHO, NAD, and GSH with a blank without GSH (L. Uotila and M. Koivusalo, to be published). The error caused by difference in molar absorptivities of NADH at 340 nm and *S*-lactylglutathione at 240 nm has been corrected. Explanation of symbols: (○) lactyl-SG; (⊡) acetyl-SG; (□) propionyl-SG; (●) succinyl-SG (1:60 in Figure 2A; 1:30 in Figure 2B); (△) formyl-SG (1:40 in Figure 2A; 1:30 in Figure 2B); (⊙) formaldehyde dehydrogenase (20:1).

Formylglutathione is not decomposed by MalNEt in these conditions since the amount of hydroxamate color was not reduced by MalNEt.

The thiol esters were stored at −20° (pH 3–6). Lactyl-, acetyl-, and propionyl-SG showed no decomposition during several months when stored in this way. On the other hand, succinyl- and formyl-SG were unstable. They could be kept 1–2 weeks at −20°. Data on the ultraviolet spectra of glutathione thiol esters is collected in Table I.

TABLE I: Ultraviolet Spectral Data for Thiol Esters of Glutathione.

Thiol Ester	$\Delta\epsilon$ (M^{-1} cm^{-1})[a]	λ_{max} (nm)[b]	ϵ_{max} (M^{-1} cm^{-1})[b]
S-Lactylglutathione	3310	233	4020
S-Glycerylglutathione	3370	235	4100
S-Glycolylglutathione	3260	233	4530
S-Mandelylglutathione	4200	235	4980
S-Acetoacetylglutathione	3400	236	4100
S-Succinylglutathione	3250	232	4740
S-Formylglutathione	3300	235[c]	3830[c]
S-Acetylglutathione	2980	230	4500
S-Propionylglutathione	3070	231	4600

[a] The figures give the molar absorptivities of the thiol ester bond at 240 nm in 0.1 M Tris-HCl buffer (pH 7.40) obtained by the methods described in text. [b] The spectra were recorded at 25° in 0.1 M Tris-HCl buffer (pH 7.40) except the spectrum of *S*-succinylglutathione which was recorded in 5 mM HCl. [c] For *S*-formylglutathione the values reported are taken from a difference spectrum obtained by enzymic hydrolysis.

The quantitative ninhydrin method (Moore and Stein, 1948) was used to test the reactivity of the free amino groups of the chemically prepared glutathione thiol esters. All tested substrates (succinyl-SG, acetyl-SG, propionyl-SG, and formyl-SG) had all amino groups free. Diketene does not react with the amino group of GSH (Decker and Lynen, 1955).

Acetyl-CoA and succinyl-CoA were prepared according to Simon and Shemin (1953). Methylglyoxal, glyoxal, phenylglyoxal, and Dowex 1 were from Fluka AG, Buchs, Switzerland. Methylglyoxal was steam distilled before use. Hydroxypyruvaldehyde was prepared and purified according to Reeves and Ajl (1965). GSH, diketene, and MalNEt were from Sigma Chemical Co., Sephadex G-100 from Pharmacia, thiolacetic acid, thiopropionic acid, and Nbs_2 from Aldrich Chemical Co., thioglycolic acid from B.D.H., and CoA (free from GSH) from P-L Biochemicals.

Assay of Glutathione Thiol Esterases. The assay mixture contained 67 mM Tris-HCl buffer (pH 7.40), 0.5 mM substrate, and enzyme in a total volume of 3.0 ml. The hydrolysis was followed after addition of enzyme at 240 nm in a Beckman DU spectrophotometer, constantly kept at 25° with a thermostat. A blank without enzyme was always included and subtracted from the rate obtained in the presence of enzyme. The molar absorptivities reported in Table I were used in calculating the activities as international units.

Results

Human liver, obtained as described in the following communication (Uotila, 1973), was used as starting material for enzymic studies. A fraction purified to the ammonium sulfate stage was further purified by Sephadex G-100 gel filtration. Four milliliters of the preparation with a protein content of about 90 mg/ml was run in a column (2.5 × 45 cm) equilib-

TABLE II: Hydrolytic Activities of Human Liver toward GSH Thiol Esters (IU/ml) after Gel Filtration with Sephadex G-100.[a]

	Sephadex G-100 Equilibrated and Eluted with 10 mM Buffer		210 mM Buffer	
	Fraction 53 (Figure 2A)	Fraction 75 (Figure 2A)	Fraction 53 (Figure 2B)	Fraction 75 (Figure 2B)
S-Lactylglutathione	0.64	0.43	0.044	1.56
S-Acetylglutathione	0.35	0.06	0.27	0.21
S-Propionylglutathione	0.21	0.08	0.005	0.33
S-Formylglutathione	29.2	0.98	38.2	1.20

[a] The activities were determined at 25° with a spectrophotometer at 240 nm. Thiol ester concentration 0.5 mM.

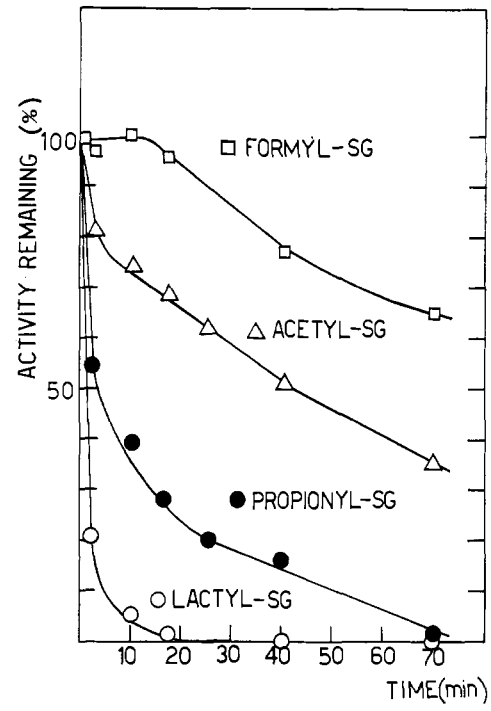

FIGURE 3: Denaturation of glutathione thiol esterases at low pH. An enzyme preparation obtained as described in the text was incubated in 0.07 M acetate buffer (pH 4.50) at 23°. At indicated times residual activity with the four substrates was measured from an aliquot by using the standard assay system.

rated and eluted with either 10 mM Tris-HCl buffer (pH 7.60) or with the same buffer containing 0.2 M KCl. The effluent was assayed for protein content (from A_{280} with an Isco UA-2 analyzer) and for hydrolytic activity toward the thiol esters which were described above. Several separate enzyme peaks were obtained (Figure 2). Activity with lactyl-SG behaved quite differently in the two runs. At low ionic strength a very broad unsymmetrical peak was obtained (Figure 2A). At high ionic strength the activity peak was sharp and was in addition eluted considerably later (Figure 2B). The same result was also obtained at low ionic strength if the sample was before run diluted with buffer to contain not more than 30 mg of protein/ml. As seen from Figure 2B, the activity peak with succinyl-SG at high ionic strength closely followed the activity with lactyl-SG, but at low ionic strength (Figure 2A) it was not transferred to another site. Glyoxalase II, which is responsible for all the activity with lactyl-SG, is in fact also active with succinyl-SG (Uotila, 1973), but the specific enzyme is very active toward succinyl-SG and so only its effect is seen in Figure 2 in which a very dilute enzyme solution was used. The peak fractions had no activity with succinyl-CoA.

With formyl-SG a highly active peak was obtained, the site of which was not influenced by change in ionic strength (Figure 2). The activity with formyl-SG was eluted in the initial part of glyoxalase II in 10 mM buffer, but was fully separated from it in 0.2 M KCl. It has been supposed previously (Strittmatter and Ball, 1955) that in the formaldehyde dehydrogenase reaction *S*-formylglutathione is formed and then hydrolyzed to formate and GSH possibly by the same enzyme. From Figure 2B it is seen that these activities are not due to the same protein but are partially separated by Sephadex G-100. Activity with acetyl-SG mostly followed activity with formyl-SG in 10 mM buffer (Figure 2A), but in 0.2 M KCl two separate peaks were obtained with acetyl-SG, one following the activity with *S*-formyl- and the other with *S*-lactylglutathione. From purification data of glyoxalase II (Uotila, 1973) it seems apparent that the second activity peak with acetyl-SG in Figure 2B is due to glyoxalase II. The first peak may be due to a side activity of the *S*-formylglutathione hydrolyzing enzyme, since the rate in these fractions was nearly 150-fold with *S*-formylglutathione compared to *S*-acetylglutathione (Table II). The fractions containing the first acetyl-SG peak of Figure 2B had no activity toward acetyl-CoA.

Activity peaks obtained with propionyl-SG are also shown in Figure 2. They followed rather closely the pattern obtained with lactyl-SG. However, the ratio of activities with lactyl- and propionyl-SG was higher in the last part of the broad peak of Figure 2A than in the initial part. As is seen from Figure 2B, the fractions which contained activity toward formyl-SG and the first peak with acetyl-SG had no activity with propionyl-SG.

All the other thiol esters studied, *S*-mandelyl- *S*-glyceryl-, *S*-glycolyl- and *S*-acetoacetylglutathione, gave in these experiments activity peaks closely coinciding with the activity toward *S*-lactylglutathione.

The properties of the thiol esterases were further studied by denaturation experiments. For those studies the activity peak with *S*-formylglutathione shown in Figure 2A was pooled. It is seen from Figure 3 that when the pooled preparation was incubated in acid pH as described in the legend of the figure the activity ratios with tested substrates were considerably changed. Activity with lactyl-SG rapidly disappeared altogether. With acetyl-SG and propionyl-SG a rapid initial fall was seen and then a slower decline. The rapid fall is apparently due to glyoxalase II. After 70 min the activity with propionyl-SG also disappeared, while part of the activity with acetyl- and formyl-SG was still present. In Figure 4 an incubation experiment in alkaline pH is presented. The order of denaturation of activities was reversed in these conditions compared to acid pH. In the experiment of Figure 5 the mixture was heated at 55°. The result was qualitatively the same as in alkaline pH.

In Table III the effect of EDTA on glutathione thiol esterases is shown. A protein fraction from human liver precipitated with ammonium sulfate (35–70% of saturation) was used. No differences in relative activities with lactyl-, acetyl-, and propionyl-SG were noted after short storage without

TABLE III: Effect of EDTA on Glutathione Thiol Esterases of Human Liver.[a]

	Initial	20 hr	4 days	7 days	10 days	3 months
			A. Without EDTA			
S-Lactylglutathione	100	99.0	94.2	83.5	84.5	25.3
S-Acetylglutathione	25.4	25.2	19.9	18.0	18.0	2.50
S-Propionylglutathione	23.0	22.6	18.6	17.1	16.1	2.73
			B. With 10 mM EDTA			
S-Lactylglutathione	102	91.0	58.8	35.3	23.1	0
S-Acetylglutathione	25.6	21.2	17.3	12.8	11.4	0
S-Propionylglutathione	22.7	22.1	12.3	8.85	6.45	0

[a] The solutions with and without EDTA were stored at 0° in 0.01 M Tris-HCl buffer (pH 7.60). Initial activity with *S*-lactylglutathione without EDTA was set to 100. The rates with different substrates are compared on a molar basis.

EDTA at 0°, but after long-term storage the ratios recall those of purified glyoxalase II (Uotila, 1973). When the solution contained 10 mM EDTA, the activities became unstable (Table III) even if no instantaneous effect was seen. The activities with lactyl-SG and propionyl-SG were lost rather similarly, but the activity with acetyl-SG appeared to be more resistant to EDTA.

Discussion

The results of this study indicate that in addition to glyoxalase II at least two glutathione thiol esterases are found in human liver: *S*-succinylglutathione hydrolase and *S*-formylglutathione hydrolase, both of which were chromatographically separated from glyoxalase II. The denaturation experiments at low pH, high pH, and high temperature all suggested that part of the hydrolysis of *S*-acetyl- and *S*-propionylglutathione is distinct from glyoxalase II, and more evidence has been obtained during purification of the latter (Uotila, 1973). This, together with the purification data of *S*-formylglutathione hydrolase and *S*-succinylglutathione hydrolase (to be published), is suggesting that part of the hydrolytic activity of liver homogenate toward *S*-acetyl- and especially *S*-propionylglutathione is separate from all purified sources. Therefore, still a fourth activity seems to be present but confirmation on this point awaits purification of the possible additional hydrolase.

The first activity peak with *S*-acetylglutathione in Figure 2B probably presents the same activity as was described by Kielley and Bradley (1954) from mouse liver. In denaturation experiments at low pH and high temperature the activities with lactyl-SG and acetyl-SG were qualitatively similar to those in the experiments of Kielley and Bradley. However, the differences were not as absolute as in the experiments of these authors where almost all activity with acetyl-SG was retained in acid pH while all glyoxalase II activity disappeared and the opposite result was noted at 55°. The latter result seems impossible with human liver, since purified glyoxalase II still has

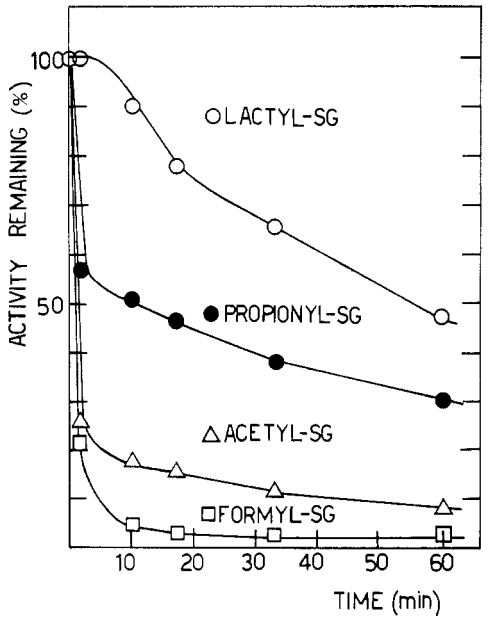

FIGURE 4: Denaturation of glutathione thiol esterases at high pH. Incubation in 0.07 M $NaHCO_3$-Na_2CO_3 buffer (pH 10.4). Other details as in Figure 3.

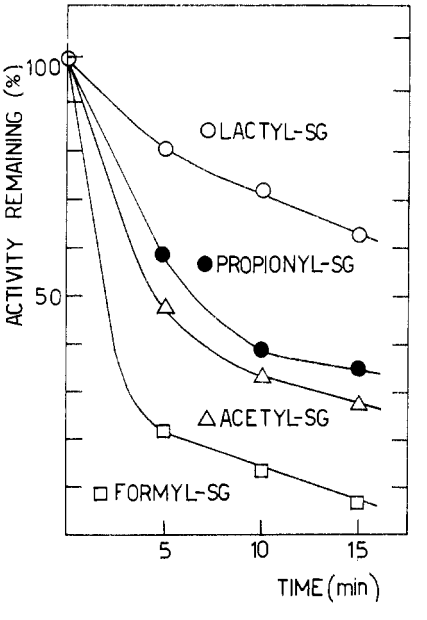

FIGURE 5: Denaturation of glutathione thiol esterases at 55°. Enzyme solution obtained as in Figures 3 and 4 incubated in 0.05 M Tris-HCl buffer (pH 7.40). At indicated times an aliquot was taken and centrifuged after rapid cooling. Residual activities measured from supernatants.

activity toward acetyl-SG (Uotila, 1973). The results in disagreement with this study obtained in denaturation experiments by Wieland *et al.* (1956) and Drummond and Stern (1961) remain to be explained. Both of these studies were made with beef liver, and species differences are by no means excluded. However, preliminary experiments have suggested that the new enzymes described in this report are not limited to human liver.

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