Purification and Characterization of *S*-2-Hydroxyacylglutathione Hydrolase (Glyoxalase II) from Human Liver†

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ABSTRACT: *S*-2-Hydroxyacylglutathione hydrolase (glyoxalase II) from human liver has been purified 4300 times compared to the 23,000*g* supernatant of liver homogenate. The purest preparation catalyzes the hydrolysis of 822 μmol of *S*-lactylglutathione per minute per mg of protein at 25° and contains one major and one minor band in disc gel electrophoresis. Hydrolytic activities toward nine glutathione thiol esters have been followed throughout purification. All are hydrolyzed by the purest enzyme preparation, but the relative activities toward *S*-formyl-, *S*-succinyl-, *S*-acetyl-, and *S*-propionylglutathione compared with the hydrolytic rate of *S*-lactylglutathione are diminished during purification. Purified glyoxalase II has no activity with thiol esters of coenzyme A or thioglycolate, carboxyl esters, or *p*-nitrophenyl acetate. Human liver glyoxalase II is a basic protein with a p*I* of 8.35 determined by isoelectric focusing. An estimate of the molecular weight by gel filtration gave a value of 22,900. The purified enzyme is unstable, but is stabilized by bovine serum albumin, glycerol, and low molecular weight thiols. Rather high concentrations of SH inhibitors are required to block the enzyme activity. Diisopropyl phosphorofluoridate is not inhibitory. 2,4,6-Trinitrobenzenesulfonate inactivates the enzyme especially at high pH. Glyoxalase II is also inhibited by the hemimercaptal formed from GSH and methylglyoxal, GSH, and several anions. EDTA has no instantaneous effect but causes slow inactivation of the enzyme.

The results presented in the first report of this series (Uotila, 1973) indicated the presence in human liver preparations of several enzymes with hydrolytic activity toward thiol esters of glutathione. The only glutathione thiol esterase which previously has been convincingly established is *S*-2-hydroxyacylglutathione hydrolase (glyoxalase II, EC 3.1.2.6). The best reported purifications of this enzyme have been only 20–30-fold (Racker, 1951; Drummond and Stern, 1961; Jerzykowski *et al.*, 1968), and it is not known whether other glutathione thiol esterases were still present in these preparations.

In the present communication a 4300-fold purification of glyoxalase II is described from human liver. The preparation obtained contains only one impurity according to disc gel electrophoresis and is free from other glutathione thiol esterases. The purified enzyme still has activity toward all tested glutathione thiol esters, but the specificity differs from that obtained with a crude preparation.

Materials and Methods

The synthesis, purification, and assay of thiol esters of GSH[1] and CoA were described earlier (Uotila, 1973). The thiol esters of thioglycolate were prepared by using the respective acid anhydrides as acylating agents (Simon and Shemin, 1953). *S*-Hexylglutathione was synthesized and crystallized according to Vince and Wadd (1969). The crystals were analytically pure (free amino group analysis compared to a calculated value). *S*-Methylglutathione, HgBzOH, *p*-nitrophenyl acetate, and 2,4,6-trinitrobenzenesulfonate were from Sigma Chemical Co. Hydroxylapatite (Hypatite C) was from Clarkson Chemical Co. The ampholytes for isoelectric focusing were from LKB, Stockholm. The standard proteins for molecular weight determination were from Boehringer. Diisopropyl phosphorofluoridate was obtained from Eastman.

Determination of Enzyme Activity. Gloxalase II activity was measured either by following the decrease of absorbance at 240 nm as described previously (Uotila, 1973), or alternatively by following the formation of GSH by the increase of absorbance at 412 nm in an assay mixture containing 67 mM Tris-HCl buffer (pH 7.40), 0.5 mM substrate, and 0.15 mM Nbs_2. A blank without enzyme was always included. The assay cuvette was thermostated at 25°. One unit of enzyme catalyzes the hydrolysis of 1 μmol of *S*-lactylglutathione/min in these conditions. At 240 nm a molar absorption coefficient of 3300 cm^{-1} was used for *S*-lactylglutathione (Racker, 1951; Cliffe and Waley, 1961; Uotila, 1973) and for other substrates the values previously reported (Uotila, 1973) were used. With the Nbs_2 method the same molar absorption coefficient, 13,600 cm^{-1} (Ellman, 1959), was used for all substrates. Glyoxalase II is not inhibited by the amount of Nbs_2 used (see below).

Protein was measured from the effluent of chromatography columns from absorbance at 280 nm and otherwise by the method of Lowry *et al.* (1951) except in the initial stage, where the biuret method was used (Gornall *et al.*, 1949). The cloudy mixture was cleared by 0.2% sodium desoxycholate (final concentration). In the last two stages of purification of glyoxalase II the samples used for protein determination were concentrated severalfold in an Amicon ultrafiltrator with a PM-10 membrane. Thiols and glycerol were first removed by dialysis.

Hydrolysis of thiol esters of CoA and thioglycolate was measured at 232 nm in a similar mixture as with thiol esters of glutathione. Hydrolysis of carboxyl esters was measured ac-

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[1] Abbreviations used are: GSH, glutathione (reduced form); acyl-SG, *S*-acylglutathione; CoA, coenzyme A; acyl-CoA, *S*-acylcoenzyme A; HgBzOH, *p*-hydroxymercuribenzoate; MalNEt, *N*-ethylmaleimide; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); ME, mercaptoethanol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate.

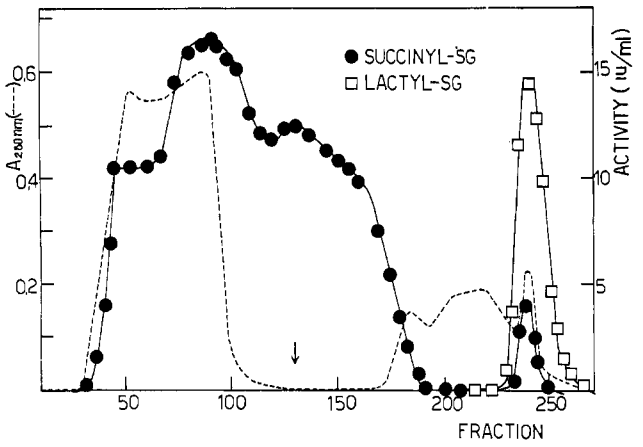

FIGURE 1: Purification of glyoxalase II by CM-cellulose chromatography (step IV). Protein measured by an analyzer with 0.33-cm cell. Between fractions 130 and 270 a linear KCl gradient was run (see text).

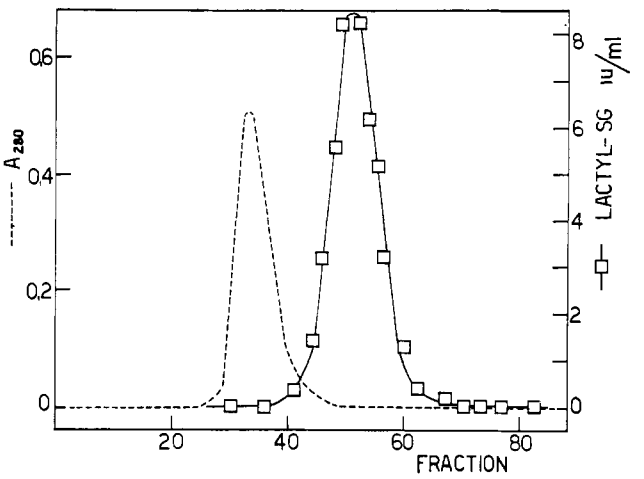

FIGURE 2: Sephadex G-75 gel filtration of glyoxalase II (step VI).

cording to Hestrin (1949). Hydrolysis of *p*-nitrophenyl acetate was determined according to Kezdy and Bender (1962).

Disc electrophoresis was performed according to the general instructions of Davis (1964). The acidic system of Maurer (1968) (pH 4.3, 7.5% acrylamide) was used. Staining was done with Amido Black.

Isoelectric focusing was performed in the 110-ml column of LKB (Model 8101) according to the instructions of the manufacturer. The sample was added in the light solution. A pH gradient 7–10 was used with 1% (w/v) ampholyte concentration. Focusing was continued 72 hr with 450 V.

Molecular weight determinations were made with a calibrated Sephadex G-100 column according to Andrews (1964). The standards used for calibration of the column were cytochrome *c* (mol wt 12,400), chymotrypsinogen (25,000), bovine serum albumin (67,000), aldolase (150,000), and catalase (230,000–250,000). The void volume was determined by Blue Dextran (2×10^6).

Purification of Glyoxalase II from Human Liver. The livers used were obtained from autopsies of persons of both sexes killed in accidents. The time between death and preparation of tissue varied from 7 to 30 hr. Freshly homogenized tissue was used as a rule, but tissue kept at −20° for some weeks gave analogous results. All steps were made at 0–4°.

Step 1. The tissue from 600 to 700 g of liver was homogenized in portions with a Waring Blendor for 2 min at 0° with 3 volumes of 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol. The homogenate was filtered through a gauze and the filtrate centrifuged at 23,000*g* for 60 min.

Step 2. Ammonium Sulfate Fractionation. To the supernatant solid, ammonium sulfate (19.4 g/100 ml) was added during 30 min. The pH was constantly kept at 7.4 with 2 M NH_3. The mixture was then gently mixed for 30 min, and was thereafter centrifuged for 30 min at 23,000*g* in a Sorvall RC-2B refrigerated centrifuge. To the supernatant more ammonium sulfate (25.0 g/100 ml) (initial volume) was added. Addition and mixing were as above; the mixture was allowed to stand at 0° overnight and was then centrifuged as above. The precipitate was dissolved in a small amount of 10 mM Tris-HCl buffer (pH 7.60) containing 2 mM ME. It was dialyzed against the same buffer for 24 hr with several changes of dialysis buffer and was then freed from insoluble material by centrifuging 10 min at 23,000*g*.

Step 3. Chromatography on DEAE-cellulose. The supernatant was put on a DEAE-cellulose column (DE-22, 5.0 × 85 cm) equilibrated with 10 mM Tris-HCl–2 mM ME (pH 7.60). The column was washed with equilibration buffer at a rate of 140 ml/hr, and 14-ml fractions were collected. Glyoxalase II activity as assayed by lactyl-SG was loosely adsorbed in these conditions to DEAE-cellulose as indicated by retarded elution and was thus half-freed from the main peak of unbound proteins and also from the activity with succinyl-SG which followed the main protein peak. Most of the purification in this stage was, however, acquired through the strong binding of a large part of the proteins. The effluent was rapidly analyzed and the active fractions were combined. The solution was freed from some turbidity by centrifugation.

Step 4. Chromatography on CM-cellulose. The supernatant was put on a CM-cellulose column (CM-22, 4.0 × 40 cm) equilibrated with the same buffer as the preceding DEAE-cellulose column. After sample application the column was washed with initial buffer until unbound proteins had been eluted from the column (analyzed from A_{280} with a Uvicord II, Figure 1). Then a linear gradient, 2 × 1000 ml, was run from initial buffer to the same buffer containing 0.12 M KCl. The enzyme was eluted in the last part of the bound proteins (Figure 1). Fractions having at least one-third of the maximum activity were pooled.

Step 5. Ultrafiltration. The solution was concentrated in an Amicon ultrafiltrator with a PM-10 membrane to one-tenth of the original volume. Slight turbidity which developed was removed by centrifugation. No activity was lost in ultrafiltration if a membrane with rapid flow and only slow mixing was used.

Step 6. Gel Filtration. The concentrate was run in Sephadex G-75 columns, 2.5 × 45 cm, medium grade. A 3.0-ml load was used with upward flow. The columns were equilibrated and eluted with 10 mM potassium phosphate (pH 7.0) containing 2 mM ME. Fractions of 2 ml were collected. The enzyme was very effectively purified in this step since most of the proteins were eluted as a single peak considerably before the enzyme peak (Figure 2). Glycerol was added to the enzyme pooled from several columns to a 30% (v/v) final concentration for stabilization.

Step 7. Hydroxylapatite Chromatography. The enzyme solution, in 7 mM potassium phosphate, 30% glycerol, and 1 mM dithiothreitol (pH 7.0), was put to a hydroxylapatite column (Hypatite C, lot 6426, 1.0 × 18 cm) equilibrated with the same solution in which the enzyme was. After the sample from two Sephadex columns had been applied, a linear gra-

TABLE I: Purification of *S*-2-Hydroxyacylglutathione Hydrolase from Human Liver.[a]

Step	Vol (ml)	Total Act. (Units)	Total Protein (mg)	Sp. Act. (Units/mg)	-fold Purified	Yield (%)
I. 23,000*g* supernatant of homogenate	1905	12,700	66,700	0.190	1.0	100
II. Ammonium sulfate (35–70%)	500	12,100	44,000	0.275	1.45	95.3
III. DEAE-cellulose	768	5,500	4,300	1.28	6.75	43.3
IV. CM-cellulose	342	3,750	370	10.1	53.2	29.5
V. Ultrafiltration	34	3,700	346	10.7	56.4	29.1
VI. Sephadex G-75	318	2,110	9.60	220	1160	16.6
VII. Hydroxylapatite	443	1,265	1.54	822	4330	10.0

[a] 630 g of liver tissue was used as starting material. For definition of enzyme unit see text.

dient from 7 to 60 mM potassium phosphate (pH 7.0) was run, 2 × 100 ml, 30% glycerol and 1 mM dithiothreitol in solutions. Thereafter the column was washed with 300 mM potassium phosphate containing 30% glycerol. The enzyme was eluted by the gradient while most impurities were not unbound before the 300-mM step.

Results

The purification of glyoxalase II is summarized in Table I. The final preparation contained only one major band in disc gel electrophoresis and in addition a very fine impurity (Figure 3).

Isoelectric Focusing. A single symmetrical peak corresponding to a p*I* of 8.35 was obtained when an enzyme preparation from Sephadex G-75 stage was focused (Figure 4).

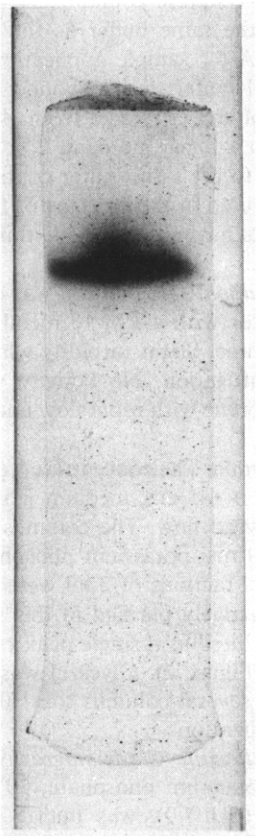

FIGURE 3: A photograph of disc gel electrophoresis of purified glyoxalase II (step VII). Run from top to bottom, anode top. Stained with Amido Black.

A single peak with the same pI was also obtained for a crude preparation (from step 2 of purification of glyoxalase II).

Substrate Specificity. The relative activities with different glutathione thiol esters were followed throughout purification (Table II). With glycolyl-, mandelyl-, glyceryl-, and acetoacetyl-SG the activity ratios to lactyl-SG were remarkably constant throughout the 4300-fold purification. Therefore, it seems apparent that the hydrolysis of all these substrates, including lactyl-SG, is catalyzed only by glyoxalase II in human liver.

In contrast part of the activity with formyl-, succinyl-, acetyl-, and propionyl-SG was lost during purification of glyoxalase II. With the first two the change was very marked. With formyl-SG a considerable loss of activity occurred in DEAE-cellulose chromatography owing to removal of a specific *S*-formylglutathione hydrolase (Uotila, 1973). After DEAE-cellulose chromatography the activity ratio between lactyl-SG and formyl-SG was quite constant (Table II). With succinyl-SG part of the loss occurred in DEAE-cellulose step and the rest in CM-cellulose step (Figure 1) owing to removal of a specific *S*-succinylglutathione hydrolase (Uotila, 1973). In contrast, no activity peaks with acetyl- or propionyl-SG were ever found in DEAE- or CM-cellulose chromatography separate from the peak of glyoxalase II. There is some reason to think that these lost activities were eluted together with glyoxalase II mostly in inactive form, since the relative activities with acetyl- and propionyl-SG were constantly somewhat higher after DEAE and CM steps than from the Sephadex G-75 stage onwards (Table II).

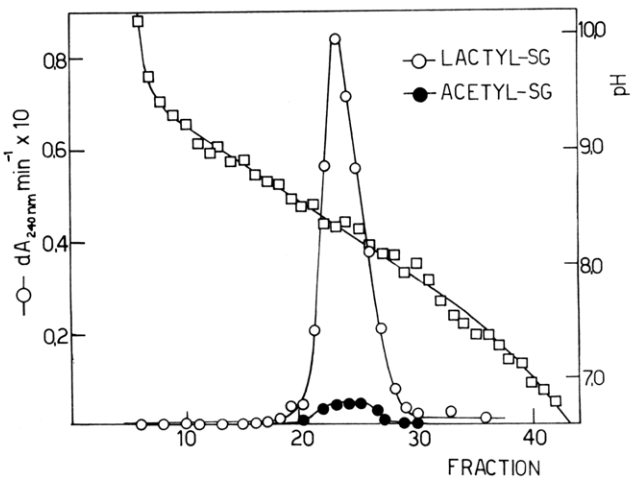

FIGURE 4: Isoelectric focusing of glyoxalase II from Sephadex G-75 stage (step VI). The protein content was too low for analysis.

TABLE II: Relative Activities toward Glutathione Thiol Esters during Purification of Glyoxalase II.[a,b]

	23,000*g* Supernatant	Ammonium Sulfate	DEAE-cellulose	CM-cellulose	Sephadex G-75	Hypatite C	Isoelectric Focusing[c]
S-Lactylglutathione	100	100	100	100	100	100	100
S-Acetylglutathione	32.0	29.6	12.4	12.1	7.4	8.1	7.9
S-Propionylglutathione	33.2	29.7	20.2	19.0	12.7	11.8	13.7
S-Succinylglutathione	2170	1820	880	33.7	27.5	28.2	N.d.
S-Mandelylglutathione	7.2	6.4	N.d.	N.d.	5.1	7.2	N.d.
S-Glycolylglutathione	52.2	40.5	46.7	51.5	45.7	44.3	N.d.
S-Glycerylglutathione	N.d.	64.4	N.d.	N.d.	73.6	68.6	N.d.
S-Formylglutathione	1240	1220	41.2	N.d.	N.d.	37.0	44.8
S-Acetoacetylglutathione	48.5	N.d.	N.d.	47.2	52.0	49.7	N.d.

[a] Activity with *S*-lactylglutathione has been set to 100 at every stage. Comparison is made on a molar basis. [b] N.d. = not determined. [c] Focused enzyme was previously purified to G-75 stage.

All of the following thiol esters were inactive as substrates of purified glyoxalase II: *S*-formyl-, *S*-acetyl-, *S*-propionyl-, *S*-succinyl- and *S*-acetoacetylthioglycolate, acetyl-CoA, and succinyl-CoA. Carboxyl esters (ethyl acetate, ethyl glycolate) and *p*-nitrophenyl acetate were also completely inactive.

Determination of Molecular Weight. By using a calibrated Sephadex G-100 column an apparent molecular weight of 22,900 was obtained for the purified glyoxalase II in good accordance with the results of Jerzykowski *et al.* (1968) for glyoxalase II from several animal tissues.

Michaelis Constants and Maximum Velocities of Substrates (Table III). All nine glutathione thiol esters followed Michaelis–Menten kinetics in the studied concentration range (0.005–0.5 mM). Lactyl-SG had the greatest maximum velocity, but mandelyl-SG had the lowest K_m. With most substrates K_m determination was only made with the purest enzyme preparation, but for lactyl-SG the determination was made with all stages of purification after CM-cellulose step. The results were identical. Also both assays (see Methods) gave closely agreeing values. With the Nbs_2 assay it was possible to study substrate concentrations above 0.5 mM also. The reaction velocity increased up to 0.7–0.8 mM but began then slowly to decrease, probably owing to substrate inhibition. Attempts to demonstrate the catalysis of a reverse reaction from GSH and DL-lactate in the presence or absence of ATP failed.

TABLE III: Michaelis Constants and Maximum Velocities of Substrates of Purified Glyoxalase II.[a]

	K_m (μM)	V_{max} (rel)
S-Lactylglutathione	190	100
S-Glycerylglutathione	109	61.8
S-Glycolylglutathione	70	38.6
S-Mandelylglutathione	16.4	4.8
S-Acetoacetylglutathione	295	55.6
S-Succinylglutathione	200	29.2
S-Formylglutathione	153	37.7
S-Acetylglutathione	266	8.6
S-Propionylglutathione	213	13.5

[a] The plot of Lineweaver and Burk (1934) was used. Maximum velocity from the plot with lactyl-SG set to 100. Comparison is made on a molar basis.

Effect of pH on the Enzyme. The enzyme had a rather broad pH optimum at 6.8–7.5. Above pH 7.5 the activity decreased rapidly. Below pH 6.8 a slow decrease of activity was seen up to pH 4.0. Then a rapid loss of activity occurred. A study on pH stability of the enzyme showed that the rapid loss of activity at pH 4.0 was due to denaturation of the enzyme. In contrast the decrease of activity on the alkaline side was due to dissociation in the active center of the enzyme since glyoxalase II resisted a short exposure to alkaline pH at least up to pH 11.0.

Thermostability. In homogenate, glyoxalase II completely resisted heating at 55° for 15 min at pH 7.6. Purified preparations were considerably less stable. When a preparation from CM-cellulose stage was exposed to various temperatures for 5 min at neutral pH the enzyme was stable only up to 35°. Half of the activity was lost at 45° and all activity at 60°. The losses with lactyl-SG, succinyl-SG, and acetoacetyl-SG correlated very well, which suggests that all activity toward these substrates was in this stage due to glyoxalase II. An attempt was also made to change the substrate specificity of a preparation from step VI by incubation at high pH (0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 10.4) at 23°). However, losses with all glutathione thiol esters were the same, probably because only glyoxalase II was present in this stage.

TABLE IV: Effect of Low Molecular Weight Thiols on Stability of Purified Glyoxalase II at 4°.[a]

Addition	Initial	2 days	9 days	15 days
Control	100	71.5	38.2	22.5
0.18 mM dithiothreitol	100	82.1	39.3	23.2
0.91 mM dithiothreitol	99.6	80.5	73.2	61.5
5.5 mM dithiothreitol	101	83.5	74.6	65.3
0.91 mM ME	100	82.1	40.4	25.0
2.7 mM ME	102	86.9	44.7	24.6
9.1 mM ME	103	89.0	72.9	59.0

[a] An enzyme preparation from G-75-stage was used. Control was stored in 0.01 M potassium phosphate buffer (pH 7.0). Protein concentration 25 μg/ml. The initial activity without added thiol has been set to 100.

TABLE V: Effect of Methylglyoxal and GSH on Purified Glyoxalase II.

Assay Buffer	Addition		Velocity		Inhibn (%)
0.1 M Imidazole–HCl (pH 7.4)	None	$-E^a$	7.2	56.6	
		$+E^a$	63.8		
	8 mM methylglyoxal	$-E^a$	32.4	31.8	44
		$+E^a$	64.2		
	40 mM methylglyoxal	$-E^a$	79.6	21.9	61
		$+E^a$	101.5		
0.1 M Imidazole–HCl (pH 6.6)	None		59.2		
	4 mM methylglyoxal		59.2		0
	8 mM methylglyoxal		52.0		12
	40 mM methylglyoxal		56.2		5
	1.0 mM GSH		59.5		0
	1.7 mM GSH		53.2		10
	3.3 mM GSH		46.5		21
	1.7 mM GSH, 6.7 mM methylglyoxal		12.9		78
	3.3 mM GSH, 6.7 mM methylglyoxal		0		100
	6.7 mM GSH, 3.3 mM methylglyoxal		0		100

[a] Without and with enzyme, respectively. No reaction was seen in any case without enzyme at pH 6.6. The velocities are expressed as $dA\ min^{-1} \times 10^3$ at 240 nm.

Stability of the Enzyme. In crude solutions the enzyme was stable for several weeks at −20°. During purification it became increasingly unstable. After gel filtration the enzyme became sensitive to freezing (about 50% lost in one freezing and thawing), dialysis (50% lost in 8 hr), mixing, and glass surfaces compared to plastic. The purified glyoxalase II was much better stored at 0° than frozen if no stabilizer was present. Bovine serum albumin (1 mg/ml) and glycerol (30%, v/v) were very good stabilizers. They both removed the sensitivity of the enzyme to freezing. Also mercaptoethanol and dithiothreitol stabilized, but rather high concentrations were needed, especially in long-term storage (Table IV). The enzyme from the hydroxylapatite stage, purified without glycerol in the last step with only 10–15% yield, lost almost all activity in freezing. In 30% glycerol it resisted freezing, but a constant decline of activity, 30% in the first week and then slower, was seen both at 0° and at −20°.

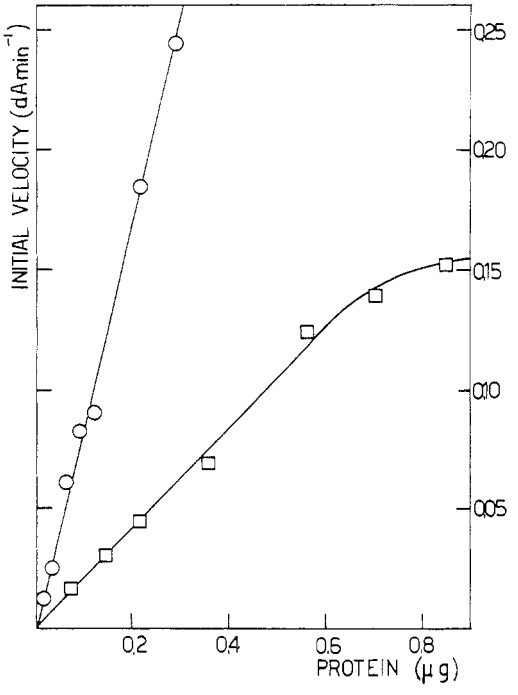

FIGURE 5: Dependence of glyoxalase II activity on protein concentration: (□) measured from decrease of absorbance at 240 nm, (○) measured with the Nbs_2 method (see text) at 412 nm.

Dependence of Activity on Enzyme Concentration. The activity followed protein concentration linearly at a rather broad range (Figure 5). With *S*-lactylglutathione as substrate the rate measured at 240 nm was linear up to an absorbance change of 0.12/min (in a cuvette of 1-cm light path). With the Nbs_2 method the rate was linear at least up to $dA = 0.25$/min (Figure 5). This result suggests that the chemical reaction following the enzymatic one in the assay with Nbs_2 is rapid enough in assay conditions not to become rate limiting.

Inhibitors. Chelating Agents. Organic models have suggested the role of metals in reactions in which thiol esters are involved (Schwyzer and Hürlimann, 1954). EDTA had, however, no instantaneous effect on enzyme from any purification stage. However, inclusion of 1 mM EDTA to buffers labilized the enzyme in long-term storage. Reactivation was tried with several metals (Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}) without success. Also EGTA (up to 5 mM) and 8-hydroxyquinoline (0.05 mM) were without effect after 30-min incubation with the enzyme.

Ions. The following metal salts had no effect: $MgSO_4$, $MnSO_4$, $NiCl_2$, zinc acetate, $Co(NO_3)_2$, all 1 mM; copper acetate (0.1 mM). NaCl, KCl, and NH_4Cl gave some inhibition above 15 mM. All three inhibited 55% at 0.4 M and 75% at 1 M. Fluoride was without effect up to 40 mM. 20 mM oxalate caused 38% inhibition and 40 mM oxalate 80%. 67 mM phosphate inhibited 35% and 167 mM 65%. Arsenate had a similar effect. 5 mM pyrophosphate inhibited 20% and 25 mM 50%.

S-Methylglutathione (1 mM) and *S*-hexylglutathione (0.1 mM) were not inhibitory when added to the standard assay system. Glyoxalase II is not sensitive to organophosphates since diisopropyl phosphorofluoridate up to 2 mM caused no inhibition.

Aldehydes and GSH. Formaldehyde (10 mM) and acetaldehyde (10 mM) were without effect. Ketoaldehydes, like methylglyoxal and glyoxal, had little or no effect in 0.1 M

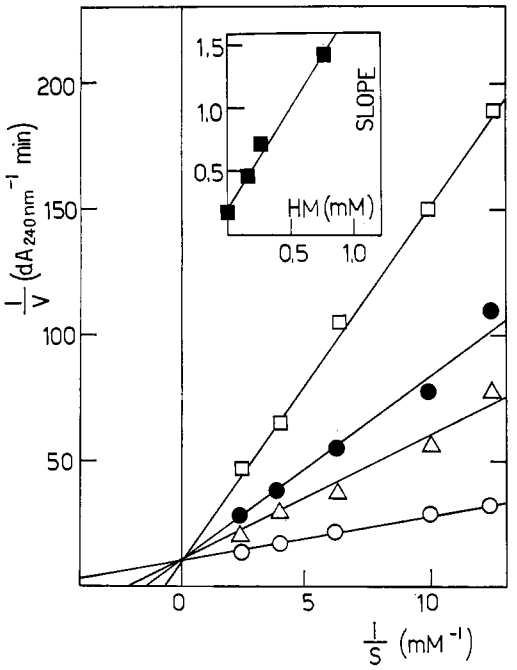

FIGURE 6: Inhibition of glyoxalase II (step VII) by the hemimercaptal of GSH and methylglyoxal in 0.1 M imidazole buffer (pH 6.60). The concentrations of GSH and methylglyoxal were 0 (○), 0.7 mM (△), 1.0 mM (●), and 2.0 mM (□) (an equimolar mixture was used), from which the respective hemimercaptal (HM) concentrations were calculated according to Cliffe and Waley (1961). The inset shows that the competitive inhibition obtained is a linear function of inhibitor concentration.

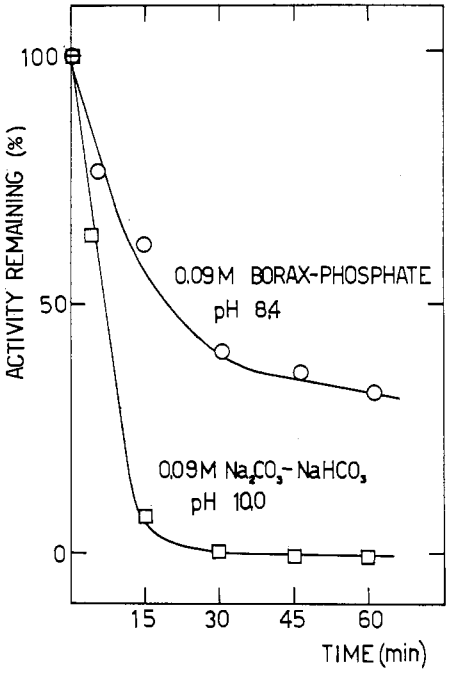

FIGURE 7: Inactivation of glyoxalase II by 2,4,6-trinitrobenzenesulfonate (0.9 mM) at 23°. The points show the residual activity compared to a similar control without 2,4,6-trinitrobenzenesulfonate.

imidazole buffer (pH 6.6) (Table V). However, marked inhibition was seen with high concentrations in the same buffer at pH 7.4. Since spontaneous hydrolysis of lactyl-SG was much increased (Table V) by methylglyoxal in this pH, it is possible that interaction of the ketoaldehyde with substrate caused part or all of the inhibition.

GSH was a rather weak inhibitor with competitive kinetics. The K_i determined at pH 7.4 with *S*-lactylglutathione as substrate was 4.0 mM. When both GSH and methylglyoxal were present, marked inhibition was seen at pH 6.6 with concentrations in which neither alone was inhibitory (Table V). Since the inhibition increased similarly by increasing either the concentration of GSH or that of methylglyoxal, it seems probable that the hemimercaptal spontaneously formed from GSH and methylglyoxal with a dissociation constant of 2 mM at pH 6.6 (Cliffe and Waley, 1961) was the true inhibitor. When the inhibition was studied kinetically by using *S*-lactylglutathione as substrate in 0.1 M imidazole buffer (pH 6.6), linear competitive inhibition with a K_i of 0.12 mM was noted (Figure 6).

Products of the Reaction. DL-Lactate (70 mM) caused no inhibition. The inhibition of GSH noted above may be product inhibition, but may also be due to binding of the substrate to the large excess of GSH (Kielley *et al.*, 1954).

Amino Group Inhibitors. The effect of 2,4,6-trinitrobenzenesulfonate was studied with concentrated enzyme from the CM-cellulose stage in borax–phosphate buffer (pH 8.4) and Na_2CO_3–$NaHCO_3$ buffer (pH 10.0). The solution together with a control without 2,4,6-trinitrobenzenesulfonate was incubated at room temperature. Activity was measured from small aliquots at definite times (Figure 7). 2,4,6-Trinitrobenzenesulfonate inactivated the enzyme, more rapidly and more completely at the higher pH, suggesting that more groups reacted at pH 10.0.

SH Inhibitors. The enzyme from the Sephadex G-75 stage without glycerol was used. For these studies the gel filtration step was run without mercaptoethanol in buffer to obtain a highly active preparation from which unbound inhibitor could be removed by dilution, since the enzyme of this stage lost much of its activity in dialysis or gel filtration with Sephadex G-25.

$HgCl_2$ was the most potent inhibitor (Table VI). Its difference from the same concentrations of HgBzOH was marked. Rather high concentrations of alkylating and oxidizing agents

TABLE VI: Effect of SH Reagents on Purified *S*-2-Hydroxyacylglutathione Hydrolase.[a]

Addition	Concn (M)	Inhibn (%)
$HgCl_2$	10^{-6}	9
	10^{-5}	82
	10^{-4}	99
HgBzOH	5×10^{-5}	0
	10^{-3}	29
	5×10^{-3}	100
Nbs_2	2×10^{-4}	0
	5×10^{-4}	12
	10^{-3}	30
Iodoacetate	10^{-3}	17
	5×10^{-3}	60
MalNEt	10^{-3}	24
$CdCl_2$	10^{-3}	39
AsO_2^-	5×10^{-3}	23
	14×10^{-3}	75

[a] Enzyme and inhibitor were incubated for 60 min at 0° in 0.08 M Tris-HCl buffer (pH 7.40). Activity measured from a 100–500-fold dilution. Substrate *S*-lactylglutathione. Control was always included.

TABLE VII: Reactivation of Liver *S*-2-Hydroxyacylglutathione Hydrolase Inhibited by $HgCl_2$.[a]

Addn (M)	Init Inhibn (%)	Inhibn after Reactivation for 10 hr (%)	Inhibn after Reactivation for 24 hr (%)
$HgCl_2$			
10^{-5}	82	37	33
10^{-4}	99	70	61
10^{-3}	99	91	86

[a] Enzyme and inhibitor were incubated at 0° for 60 min. After activity determinations dithiothreitol (5.4 mM) was added to tubes and incubation continued at 4°. Control was treated similarly.

were needed to cause inhibition. Slight inhibition was also noted with agents known to react with dithiols ($CdCl_2$, arsenite). The study of the effects of arsenite was complicated, since it catalyzed chemically the hydrolysis of *S*-lactylglutathione.

An attempt was made to reactivate the enzyme inhibited by $HgCl_2$ or HgBzOH with dithiothreitol (Table VII). The enzyme inhibited by low concentrations of $HgCl_2$ was clearly but not completely reactivated. The inhibition obtained with high HgBzOH concentrations was not at all reversible by dithiothreitol (not shown in the table).

Discussion

Glyoxalase II seems to be specific for GSH but rather unspecific for the acyl group of the thiol ester. The purest preparation obtained appears to be free from contaminating thiol esterases since the activity ratios with different substrates were constant in the last steps (Table II). Also attempts to change the substrate specificity of the final preparations by heating or alkali failed. It seems clear that no specific hydrolase is found in human liver for *S*-acetoacetylglutathione. The result supports the view of Drummond and Stern (1961) and is in conflict with the result of Decker (1959). The amount of glyoxalase II in human liver is low, but the activity of the purified enzyme is high. A specific activity of 822 IU/mg was obtained in this study. The highest value previously reported has been 20 IU/mg from chicken kidney (Jerzykowski *et al.*, 1968).

The enzyme has marked differences from carboxyl esterases (EC 3.1.1.1.) as indicated by basicity, lower molecular weight, inability to catalyze the hydrolysis of carboxyl esters and *p*-nitrophenyl acetate, and resistance to 2 mM diisopropyl phosphorofluoridate.

The purified enzyme was very unstable, but by using glycerol or bovine serum albumin as stabilizing agent the enzyme can be stored for a long time. The stabilizing effect of thiols and studies with SH inhibitors suggested that SH groups are found in the enzyme but too high concentrations of SH reagents were in most cases needed to cause inhibition for an SH group in the active center of the enzyme to be postulated. The result is unexpected, since the most probable mechanism would be one in which the acyl group of GSH is transferred to water through a thiol group of the enzyme. One possible explanation might be a thiol ester group between an SH and a carboxyl group in the active center of the enzyme. The finding that $HgCl_2$ but not HgBzOH was inhibitory in small concentrations is compatible with this interpretation, since the former but not the latter is often known to break thiol ester bonds (Webb, 1966). However, steric reasons can also cause a difference in the efficiency of $HgCl_2$ and HgBzOH.

The physiological significance of glyoxalase II and other glutathione thiol esterases is unknown. The following routes for enzymic synthesis of GSH thiol esters are known: lactyl-SG and some others (including α-D-hydroxyglutarylglutathione; Jerzykowski *et al.*, 1971) *via* glyoxalase I reaction; *S*-acetylglutathione by a brain enzyme from ATP, GSH, and acetate (Feuer, 1956) or by thiolysis from acetoacetyl-CoA and GSH (Stern and Drummond, 1961); *S*-formylglutathione through formaldehyde dehydrogenase reaction (Rose and Racker, 1962; L. Uotila and M. Koivusalo, unpublished experiments), and *S*-palmitylglutathione from palmityl-CoA or palmityl adenylate (Vignais and Zabin, 1958). In addition chemical synthesis of GSH thiol esters from those of CoA is possible in physiological pH (Stadtman, 1952).

It seems reasonable that other acceptors of the acyl groups of GSH than water might function *in vivo*. Transfer reactions with the nearly pure enzyme will be studied in the future. No role has been established for glutathione thiol esters in synthetic processes but little if any study on the subject has been performed.

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Characterization of Poly(riboadenylic acid) Segments in L-Cell Messenger Ribonucleic Acid†

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ABSTRACT: The polyadenylate segments in L-cell mRNA have been characterized by the use of two-dimensional "fingerprinting" techniques. Digestion of poly(A)-containing mRNA with pancreatic ribonuclease and T_1 ribonuclease in high salt buffer releases the intact poly(A) segments; these are readily separable from the (A-)nCp, (A-)nGp, and (A-)nUp components which arise by digestion from the remainder of the molecule. Digestion of the same mRNA preparations with both enzymes, but in low salt buffer, results in the hydrolysis of the poly(A) segments to oligonucleotides with chain lengths $2 \geqslant 15$; these oligo(adenylic acid) components are also largely separable from the (A-)nCp, (A-)nGp, and (A-)nUp components. Fractionation of the alkali hydrolysis products of the oligoadenylates or of poly(A) released by digestion in high salt buffer did not reveal the presence of guanylic, cytidylic, or uridylic acid. This suggests that poly(A) segments in L-cell mRNA are homopolymers of adenylic acid. Using the same methods, no poly(A) could be detected in L-cell ribosomal RNA.

Poly(riboadenylic acid) [poly(A)] segments of up to 250 nucleotides in length have been reported to be covalently linked to heterogeneous nuclear RNA, eukaryotic messenger RNA (Lim and Canellakis, 1970; Darnell *et al.*, 1971b; Lee *et al.*, 1971; Edmonds *et al.*, 1971), and viral RNA (Kates, 1970; Lai and Duesberg, 1972).

The functional role of untranslated regions in eukaryotic mRNA such as the poly(A) tracts is uncertain at this time. However, there is evidence to suggest that poly(A) may be necessary for processing and transport of mRNA from the nucleus to the cytoplasm (Darnell *et al.*, 1971a). The recent demonstration that poly(A) tracts located at the 3′ terminus are longer in newly synthesized mRNA (Greenberg and Perry 1972a; Sheiness and Darnell, 1973) indicates that this region of the RNA may also serve to protect the informational part of the molecule from nucleolytic destruction during its life span in the cell. Further, the interaction of a heterogeneous population of mRNA molecules with ribosomes during translation or possibly with proteins during transport from the nucleus to the cytoplasm (Henshaw, 1968; Samarina *et al.*, 1968; Perry and Kelley, 1968) would require invariable regions in mRNA. Regions of the RNA which could be involved in RNA–protein interactions are likely to be in the untranslated regions at the chain termini, such as the poly(A) segments. Evidence for the involvement of mRNA poly(A) segments in mRNA–protein interactions has been described (Kwan and Brawerman, 1972; Blobel, 1973).

In order to gain some insight into the structure and post-transcriptional mechanism of the addition of poly(A) to mRNA, the isolation and nucleotide sequence analysis of the poly(A) segments in L-cell mRNA have been studied. The present report suggests that poly(A) tracts in chain lengths up to about 250 residues consist of only adenylic acid residues. No regions of the mRNA adjacent to the poly(A) segment are released with the poly(A) by nuclease digestion in high salt buffer.

Materials and Methods

Cells. Mouse L-cells were grown in Eagles minimum essential medium (Joklik-modified, Grand Island Biological Co.), containing 5% fetal calf serum.

Labeling of Cells. L-Cells ($1–2 \times 10^8$) in 30-ml cultures were grown for 3 to 4 hr at 37° in the presence of [2,8-^{3}H]-adenosine (150 μCi/ml) or carrier-free $^{32}PO_4$ (175 μCi/ml). Labeling of cells in the presence of $^{32}PO_4$ was done in phosphate-free medium.

In some experiments labeling of cells was carried out after an initial 30-min incubation in the presence of actinomycin D (0.05 μg/ml, Calbiochem) or in the presence of actinomycin D (0.05 μg/ml) and ethidium bromide (1 μg/ml, Calbiochem).

Cell Fractionation and Isolation of mRNA Containing Poly(A) Segments. Breakage of cells and preparation of post-mitochondrial supernatants were accomplished by procedures described previously (Penman *et al.*, 1963; Vesco and Penman,

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