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DISULFIDE REDUCTION IN RAT LIVER

II. CHROMATOGRAPHIC SEPARATION OF NUCLEOTIDE-DEPENDENT DISULFIDE REDUCTASE AND GSH DISULFIDE TRANSHYDROGENASE ACTIVITIES OF THE HIGH-SPEED SUPERNATANT FRACTION

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SUMMARY

1. Three disulfide-reducing activities found in the high-speed supernatant fraction of rat liver, *viz.* glutathione reductase, GSH–disulfide transhydrogenase, and a NADPH-dependent reductase of broad specificity have been separated from each other by chromatography on DEAE-cellulose.

2. Like the activity present in crude extracts, the chromatographically isolated transhydrogenase appears able to catalyze the reduction of both protein and non-protein disulfide substrates by GSH in the presence of NADPH and an exogenous source of glutathione reductase. The purified enzyme appears to be quite unstable.

3. The non-specific NADPH-dependent disulfide-reducing activity obtained by DEAE-cellulose chromatography was further purified by gel filtration on Sephadex G-150, using 5,5′-dithiobis-(2-nitrobenzoic acid)(DTNB) as indicator disulfide. The relatively low activity exhibited by this fraction in the reduction of insulin and other disulfides could be greatly increased when reaction mixtures were further supplemented with small amounts of supernatant solution obtained by heat-treatment of crude enzyme preparations at 70° for 20 min. The heat-stable component active in disulfide reduction was further purified on Sephadex G-100 and completely separated from glutathione reductase, whose activity was not affected by the heat treatment. The data presented herein is considered to support earlier sepeculations on the similarity of the nucleotide-dependent disulfide-reducing activity of normal rat liver to the thioredoxin systems found in microorganisms and rat hepatoma.

INTRODUCTION

In a previous publication[1] evidence was presented to indicate that the high-speed supernatant fraction of normal rat liver contained several enzyme activities

Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

associated with the reduction of disulfide bonds of protein and non-protein substrates. These activities included, in addition to glutathione reductase, a non-specific NADPH-dependent disulfide reductase and a GSH-disulfide transhydrogenase (oxidoreductase). The present paper describes the separation and partial purification of these enzyme systems by ion-exchange chromatography and gel filtration, and the resolution of the nucleotide-dependent disulfide-reducing activity into heat-labile and heat-stable components.

MATERIALS AND METHODS

Materials

$(NH_4)_2SO_4$, "enzyme grade", was a product of Mann Research Laboratories. DEAE-cellulose (Whatman Type DE-52) was purchased from Reeve Angel. Sephadex G-100 and G-150 were products of Pharmacia. Other reagents or products have been described[1].

Chromatographic procedures

The microsomal supernatant (high-speed supernatant) fraction of rat liver was prepared as described previously[1]. Ion-exchange chromatography on DEAE-cellulose was carried out at 4°. The adsorbent was first equilibrated in batchwise fashion with 0.1 M Tris (pH 7.5) and then on the column with a starting buffer consisting of 5 mM Tris–1 mM EDTA (pH 7.5). The dialyzed protein sample was introduced onto the column and irrigated initially with starting buffer at a flow rate of 0.7 ml/min until all unadsorbed protein had been collected. A salt gradient was then initiated by introducing a 1 M solution of NaCl in starting buffer from a Mariotte flask into a 500-ml constant volume mixing chamber filled initially with starting buffer.

Gel filtration on Sephadex G-150 or G-100 was carried out by descending irrigation with Tris–EDTA buffer (pH 7.5) delivered at a pressure head of 10–20 cm of water by means of a Mariotte flask.

Enzyme assays

Spectrophotometric assays of enzyme activities in crude extracts were carried out in the balanced systems described previously[1]. When carried out on column eluates or on chromatographically purified enzyme preparations, the procedures did not generally require the use of balanced reaction mixtures since the interfering oxidative or hydrolytic reactions encountered in the unfractionated preparations were then of low order. A unit of enzyme activity is defined arbitrarily as that quantity of enzyme which produces an absorbance change of 1.0 in 4 min. Specific activity is defined as units/mg protein.

Other procedures employed in this work have been described previously[1].

RESULTS

Enzyme activities in $(NH_4)_2SO_4$ fractions

Preliminary to attempts at chromatographic separation of the disulfide-reducing activities in rat liver microsomal supernatant, the distribution of these enzymes in $(NH_4)_2SO_4$ fractions thereof was studied. The major portion of glutathione reductase

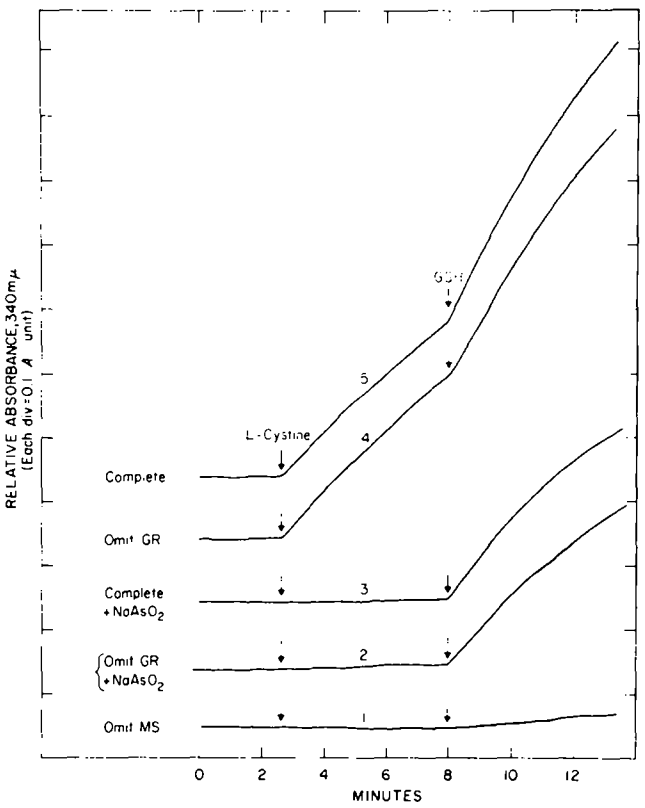

Fig. 1. Demonstration of NADPH- and GSH-dependent disulfide-reducing activities in a 40–80% $(NH_4)_2SO_4$ fraction of rat liver microsomal supernatant. Spectrophotometric measurements at 340 mμ were carried out in balanced reaction mixtures in 1.0 ml Tris–EDTA (pH 7.5). The complete system contained, at zero time, 0.2 μmole NADPH, 10 μg yeast glutathione reductase (GR), and 2.3 mg of a 40–80% $(NH_4)_2SO_4$ fraction of rat liver microsomal supernatant (MS). Sodium arsenite, 0.5 μmole, was added to both cuvettes of the indicated reaction mixtures. L-Cystine, approx. 0.5 μmole, was added to one of each pair of cuvettes and an equal volume of buffer to the other at approx. 2.5 min (first set of arrows) and 0.1 μmole GSH to both cuvettes at 8 min (second set of arrows). As explained previously[1], the relative vertical displacements of the curves are for display purposes only and are not meant to imply absolute differences in initial absorbances of the reaction mixtures.

activity was found in the 40–60% $(NH_4)_2SO_4$ fraction, while NADPH-dependent insulin- and DTNB-reducing activities were present in greatest amounts in the 60–80% fraction. GSH–hydroxyethyldisulfide transhydrogenase activity was more uniformly distributed throughout these fractions. Because of the overlapping enzyme activities in the two highest $(NH_4)_2SO_4$ fractions, all subsequent studies were carried out with the combined 40–80% $(NH_4)_2SO_4$ fraction containing over 90% of the reductase activities and more than half of the transhydrogenase activity of the high-speed supernatant fraction.

The presence of three disulfide-reducing activities in the 40–80% $(NH_4)_2SO_4$ fraction of the microsomal supernatant is shown in the spectrophotometric experiments recorded in Fig. 1. The addition of cystine (first arrow) to one of a pair of cu-

vettes balanced initially with respect to NADPH, glutathione reductase, and the 40–80% fraction (Curve 5) results in rapid oxidation of the nucleotide, which is further stimulated by the subsequent addition of $1 \cdot 10^{-4}$ M GSH (second arrow). Similar behavior is exhibited by an analogous system lacking exogenous glutathione reductase (Curve 4). The incorporation of $5 \cdot 10^{-4}$ M arsenite in either of these systems (Curves 2 and 3) suppresses completely the oxidation of NADPH seen subsequent to cystine addition but is without effect on the rate of such oxidation brought about by the addition of GSH. Curve 1 shows that the "basal" rate of oxidation of nucleotide caused by the spontaneous reduction of the disulfide substrate by added GSH in the absence of the supernatant fraction is negligible under these conditions. As indicated previously[1], these results are consistent with the presence, in the high-speed supernatant fraction of rat liver, of an arsenite-sensitive, nucleotide-dependent disulfide-reducing activity together with arsenite-insensitive glutathione reductase and GSH disulfide transhydrogenase activities. In these crude extracts the latter two activities can constitute a coupled enzyme system capable of reducing a variety of disulfide substrates.

Chromatographic separation of disulfide-reducing activities

The separation of the three disulfide-reducing activities of the 40–80% $(NH_4)_2SO_4$

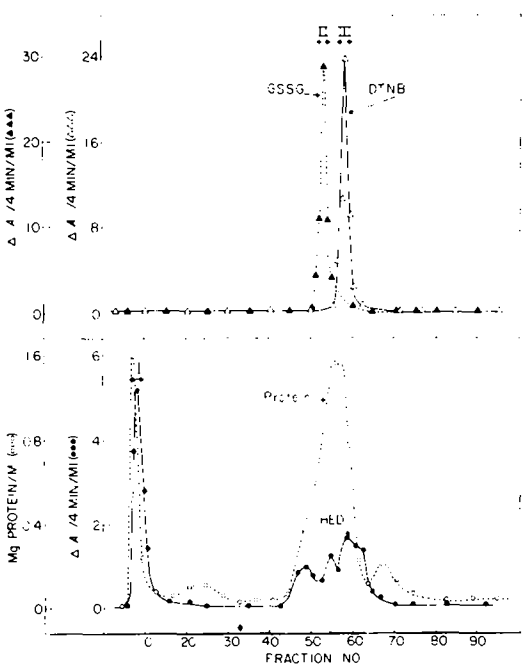

Fig. 2. Separation of GSH-hydroxyethyldisulfide transhydrogenase, GSSG reductase, and NADPH-dependent DTNB-reducing activities of the 40–80% $(NH_4)_2SO_4$ fraction of rat liver microsomal supernatant fraction (185 mg protein) on DEAE-cellulose. For details see text. Initiation of the NaCl gradient is indicated by the heavy arrow. Fractions of 6.5 ml were analyzed for protein and for GSH hydroxyethyldisulfide transhydrogenase, glutathione reductase and DTNB reductase activities by methods described previously[1]. Eluates comprising Fractions I–III were pooled, concentrated by precipitation with $(NH_4)_2SO_4$, and dialyzed against Tris EDTA buffer. HED: hydroxyethyldisulfide.

TABLE I

SPECIFIC ACTIVITIES OF DISULFIDE-REDUCING ENZYMES OBTAINED BY CHROMATOGRAPHY OF THE 40–80% $(NH_4)_2SO_4$ FRACTION OF RAT LIVER HIGH-SPEED SUPERNATANT ON DEAE-CELLULOSE

Eluate fractions from DEAE-cellulose chromatography were pooled and concentrated by $(NH_4)_2SO_4$ precipitation to yield DEAE Fractions I–III as shown in Fig. 2. Each fraction was assayed for GSH–hydroxyethyldisulfide transhydrogenase, glutathione reductase, and NADPH-dependent DTNB-reducing activity. Results are expressed as $\Delta A/4$ min per mg protein.

Substrate	*DEAE-cellulose fraction*			*Original 40–80% $(NH_4)_2SO_4$ fraction*
	I	*II*	*III*	
Hydroxyethyldisulfide	0.55	0.07	0.04	0.13
GSSG	0	2.2	0.3	3.2
DTNB	0	0	11	2.8

fraction by DEAE-cellulose chromatography is shown in Fig. 2. Associated with the sharp peak of initially unadsorbed protein is a substantial fraction of the GSH–hydroxyethyldisulfide transhydrogenase activity of the preparation. The slight retardation of enzyme activity associated with this peak has been observed in all subsequent separations. Although, in this figure, the major protein component eluted after initiation of the salt gradient (Fractions 40–65) appeared to contain three additional minor but discrete peaks of GSH–hydroxyethyldisulfide transhydrogenase activity, subsequent chromatograms showed in reality that this enzyme activity was usually spread in a more diffuse manner throughout these fractions. As can be seen, the protein fraction eluted by the salt gradient also contains two sharply defined regions of enzyme activity associated with the NADPH-dependent reductions of

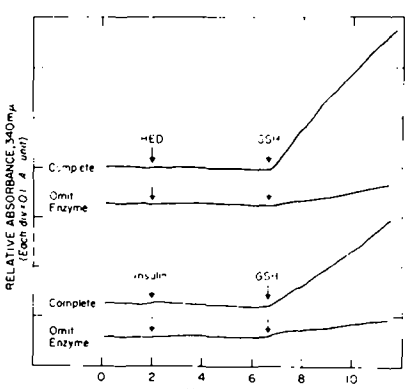

Fig. 3. Action of the chromatographically purified transhydrogenase component of the 40–80% $(NH_4)_2SO_4$ fraction of rat liver microsomal supernatant on hydroxyethyldisulfide and insulin. Transhydrogenase activity was measured spectrophotometrically at pH 7.5 in Tris-EDTA as described earlier[1]. Complete systems (1.0 ml) contained initially: 0.2 μmole NADPH, 10 μg yeast glutathione reductase, and 0.32 mg of the Fraction I component derived from DEAE-cellulose chromatography of the 40–80% $(NH_4)_2SO_4$ fraction of rat liver microsomal supernatant (Fig. 2). Hydroxyethyldisulfide (HED) (1.0 μmole), insulin (2.0 mg), and GSH (0.1 and 2.0 μmoles, respectively, for hydroxyethyldisulfide and insulin) were added as indicated. Control reductions were carried out with mixtures lacking Fraction I. Reaction mixtures were balanced with respect to all components except disulfide substrate.

GSSG and DTNB. Eluates corresponding to the foregoing enzyme activities were pooled as indicated in Fig. 2, concentrated by precipitation with $(NH_4)_2SO_4$ and dialyzed against Tris–EDTA buffer. The enzyme activities of the Fractions I–III thus obtained (Fig. 2) toward the three disulfide substrates are recorded in Table I. It is evident that each isolated fraction is essentially free of the enzyme activities characteristic of the other two fractions. The low level of transhydrogenase activity in Fractions II and III reflects the diffuse spread of this activity throughout the bulk of resin-adsorbed protein (*cf.* Fig. 2). Although the presence of a minor amount of GSSG-reducing activity in Fraction III might be ascribed to an intrinsic activity of the DTNB reductase component, the fact that this activity was largely arsenite-insensitive indicated that it was a result of slight contamination with glutathione reductase. Comparison of the specific activities of the isolated chromatographic fractions with those of the untreated 40–80% $(NH_4)_2SO_4$ preparation showed (Table I) that 4–7-fold increases in relevant enzyme activities were achieved by the ion-exchange procedure.

Fig. 3 shows that, like the crude microsomal supernatant fraction itself[1], Fraction I possessed transhydrogenase activity toward both protein (insulin) and non-protein (hydroxyethyldisulfide) disulfides. Further studies on the purification and properties of the transhydrogenase activities of this and other liver fractions are in progress.

DTNB was employed as the indicator disulfide in the chromatographic separation of nucleotide-dependent disulfide-reducing activity shown in Fig. 2 primarily because of its sensitivity as substrate. In order to determine whether the peak reductase activity observed with respect to this substrate was representative of the more general nucleotide-dependent reductase activity of the whole supernatant fraction[1] the chromatographic procedure of Fig. 2 was repeated and the resulting

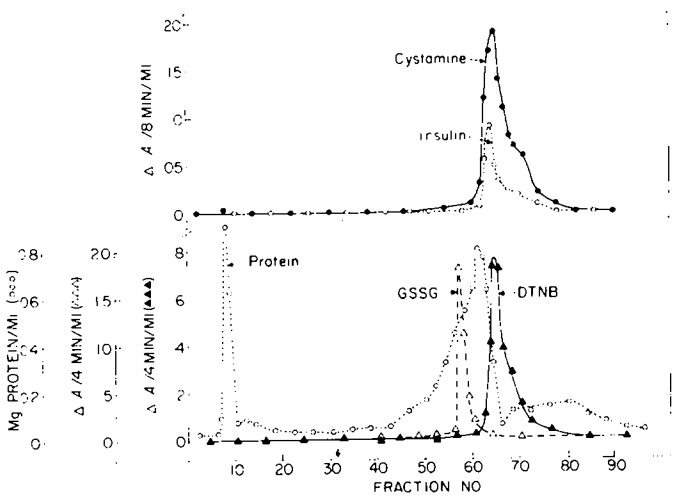

Fig. 4. Correspondence of NADPH-dependent reductions of DTNB, insulin, and cystamine in eluate fractions from chromatography of 40–80% $(NH_4)_2SO_4$ fraction of rat liver microsomal supernatant (76 mg protein) on DEAE-cellulose. For details see text. Fractions of 3.4 ml were analyzed for protein, glutathione reductase, and NADPH dependent disulfide-reducing activity with DTNB, insulin, and cystamine as substrates. Enzyme assays were carried out in reaction mixtures containing 0.2 μmole NADPH, eluate fractions, and disulfide substrates at levels of 1.0 μmole disulfide per ml. Initiation of the NaCl gradient is indicated by the heavy vertical arrow.

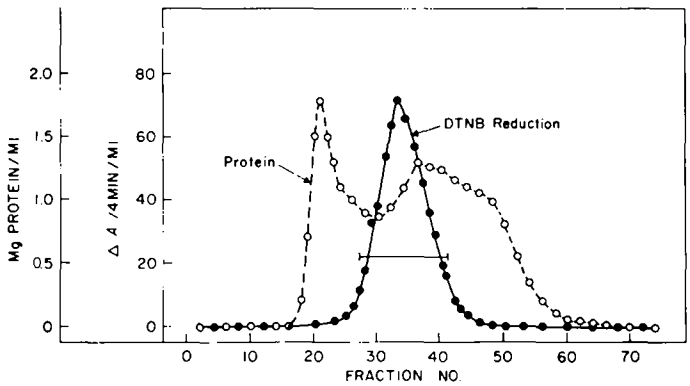

Fig. 5. Gel filtration of DEAE Fraction III on Sephadex G-150. 100 mg of Fraction III protein (Fig. 2) were dissolved in 4.5 ml Tris–EDTA and applied to a 2.5 cm × 30 cm column of Sephadex G-150. Descending irrigation was carried out at 4° at a flow rate of 0.3 ml/min. Fractions of 2.5 ml were analyzed for protein and NADPH-dependent reduction of DTNB as described previously[1].

eluates assayed for nucleotide-dependent reductions of insulin and cystamine, in addition to DTNB and GSSG. The results of this experiment, shown in Fig. 4, demonstrated complete coincidence in location of nucleotide-dependent reducing activities toward the non-glutathione substrates and indicated that the non-specific nucleotide-dependent reductions of disulfide substrates (other than GSSG) observed previously[1] in crude fractions of rat liver cytoplasm were probably catalyzed by one enzyme system.

Again using DTNB as indicator disulfide, further purification of the nucleotide-dependent disulfide reductase of rat liver supernatant was apparently accomplished by gel filtration of Fraction III on Sephadex G-150 (Fig. 5). Eluates containing DTNB-reducing activity were pooled as indicated and the concentrated product tested for NADPH-dependent reducing activity toward insulin and other disulfide substrates. Although substantial increases in enzyme activity were anticipated as a result of the chromatographic procedures shown in Figs. 4 and 5, it was found, on the contrary, that the insulin-reducing activity of these purified fractions remained at a rather low level (Table II, Expts. 1–3).

Dependence of reductase activity on a heat-stable factor

Because earlier experiments[1] on the reductase activity of unfractionated microsomal supernatant had shown evidence of stimulatory activity by heated supernatant the effect of such heated preparations on the insulin-reducing activity of the chromatographic fractions was investigated. These results showed clearly that the addition of small amounts of supernatant material obtained by treatment of the 40–80% $(NH_4)_2SO_4$ fraction of high-speed supernatant at 70° for 20 min could increase noticeably the rate of reduction of insulin by the chromatographic fractions (Table II, Expts. 4d and 5b). The addition of DEAE-cellulose Fractions I or II (Fig. 2), possessing enhanced transhydrogenase or glutathione reductase activities, respectively, was without effect on such activity (Expts. 4b and 4c). In accordance with previous findings[1] the heat-treated sample of the 40–80% $(NH_4)_2SO_4$ fraction was itself incapable of catalyzing nucleotide-dependent reduction of insulin (Table II, Expt. 4e).

TABLE II

NADPH-DEPENDENT DISULFIDE-REDUCING ACTIVITIES OF CRUDE AND PURIFIED FRACTIONS OF RAT LIVER MICROSOMAL SUPERNATANT

NADPH-dependent reduction of disulfide substrates by rat liver microsomal supernatant and purified fractions derived therefrom was followed spectrophotometrically as described earlier[1]. DEAE Fractions I–III were isolated following chromatography of the 40–80% $(NH_4)_2SO_4$ fraction of microsomal supernatant on DEAE-cellulose (Fig. 2). The Sephadex G-150 and Sephadex G-100 fractions refer to the combined and concentrated fractions obtained following gel filtration of DEAE Fraction III on Sephadex G-150 (Fig. 5) or of heat-treated (70°, 20 min) 40–80% $(NH_4)_2SO_4$ fraction of microsomal supernatant on Sephadex G-100 (Fig. 6), respectively. Specific activities listed in Expts. 1–3 represent the range of values derived from assays carried out on a number of preparations.

Expt.	*Substrate**	*Fraction*	*Addition*	*ΔA/ min per mg*
1	Insulin	40–80% $(NH_4)_2SO_4$ fraction		0.05–0.13
2	Insulin	DEAE-cellulose Fraction III		0.07–0.20
3	Insulin	Sephadex G-150 fraction		0.05–0.12
4a	Insulin	DEAE-cellulose fraction III (0.55 mg)		0.07
4b	Insulin	DEAE Fraction III (0.55 mg)	DEAE-cellulose Fraction I (0.32 mg)	0.05
4c	Insulin	DEAE Fraction III (0.55 mg)	DEAE-cellulose Fraction II (0.36 mg)	0.08
4d	Insulin	DEAE Fraction III (0.55 mg)	Heated 40–80% $(NH_4)_2SO_4$ fraction (0.20 mg)	0.34
4e	Insulin		Heated 40–80% $(NH_4)_2SO_4$ fraction (0.20 mg)	0.08
5a	Insulin	Sephadex G-150 fraction (0.18 mg)		0.05
5b	Insulin	Sephadex G-150 fraction (0.18 mg)	Heated 40–80% $(NH_4)_2SO_4$ fraction (0.20 mg)	1.4
5c	Insulin	Sephadex G-150 fraction (0.18 mg)	Sephadex G-100 fraction (0.16 mg)	0.03
6a	Rabbit γ-globulin	Sephadex G-150 fraction (0.073 mg)		0.0
6b	Rabbit γ-globulin	Sephadex G-150 fraction (0.073 mg)	Sephadex G-100 fraction (0.063 mg)	1.3
7a	L-Cystine	Sephadex G-150 fraction (0.073 mg)		0.0
7b	L-Cystine	Sephadex G-150 fraction (0.073 mg)	Sephadex G-100 fraction (0.063 mg)	1.1
7c	L-Cystine		Sephadex G-100 fraction (0.063 mg)	0.0
8a	Lipoate	Sephadex G-150 fraction (0.073 mg)		0.41
8b	Lipoate	Sephadex G-150 fraction (0.073 mg)	Sephadex G-100 fraction (0.063 mg)	2.0
9a	Cystamine	Sephadex G-150 fraction (0.073 mg)		0.57
9b	Cystamine	Sephadex G-150 fraction (0.073 mg)	Sephadex G-100 fraction (0.063 mg)	1.6

* Substrate concentrations: L-cystine, 0.25 μmole/ml; all others, 1.0 μmole disulfide/ml.

Gel filtration of heat-stable component

The purification of the heat-stable stimulatory component of the 40–80% $(NH_4)_2SO_4$ fraction of the microsomal supernatant by gel filtration on Sephadex G-100 is shown in Fig. 6. Eluates containing co-factor activity were assayed spectrophotometrically by their ability to cause nucleotide oxidation in reaction mixtures

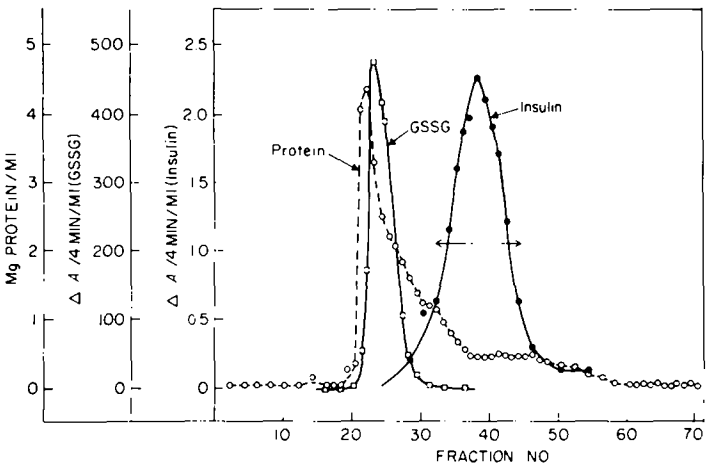

Fig. 6. Purification of the heat-stable component of rat liver microsomal supernatant by gel filtration on Sephadex G-100. Crude heat-stable protein was prepared by heating a solution of the 40–80% $(NH_4)_2SO_4$ fraction of rat liver microsomal supernatant in Tris–EDTA at 70° for 20 min. Following centrifugation, the supernatant solution was brought to 100% saturation with $(NH_4)_2SO_4$ at room temperature and the precipitated material dissolved in a small volume of buffer. 50 mg of protein was applied to a 2 cm × 20 cm column of Sephadex G-100 and irrigated at 25° with Tris-EDTA at a flow rate of 0.3 ml/min. Fractions of 1.5 ml were analyzed for protein and glutathione reductase activity as described previously[1]. Eluate fractions (50 μl) were also assayed for co-factor activity in NADPH-dependent reduction of insulin in reaction mixtures containing 0.2 μmole NADPH, 18.4 μg pooled Sephadex G-150 enzyme (Fig. 5) and 2.0 mg insulin. Active eluates were pooled as indicated and concentrated by precipitation with $(NH_4)_2SO_4$ at 100% saturation.

containing NADPH, insulin, and the Sephadex G-150 component of Fig. 5 active in DTNB reduction. The distinct separation of a fraction active in insulin reduction from the heat-stable glutathione reductase component present in the same preparation is apparent. The component active in insulin reduction was concentrated by precipitation with $(NH_4)_2SO_4$, as indicated in Fig. 6, and its participation in the reduction of a number of disulfide substrates was examined. Expts. 5–9 of Table II show that in all cases the addition of small amounts of the chromatographically isolated heat-stable factor to the (heat-labile) reductase fraction obtained by gel filtration on Sephadex G-150 (Fig. 5) resulted in significant stimulation of enzyme activity toward a variety of disulfide substrates. In a number of cases (*e.g.* Expts. 8a and 9a) noticeable reduction was obtained with the Sephadex G-150 fraction alone; this observation, together with similar activities noted elsewhere (*cf.* Figs. 4 and 8) suggest that chromatographic fractions of heat-labile material are accompanied by contaminating quantities of the heat-stable component.

Further experiments bearing on the requirement for the heat-stable factor in disulfide reduction are shown in the spectrophotometric tracings of Fig. 7. The simultaneous presence of small amounts of purified heat-labile Sephadex G-150 (Fig. 5) and heat-stable Sephadex G-100 (Fig. 6) fractions resulted in rapid oxidation of NADPH upon addition of the disulfide substrates insulin, cystine, or lipoate (Curves 4–6). In conformity with the observed behavior in the crude supernatant solutions, these activities were promptly abolished by $1 \cdot 10^{-4}$ M arsenite. Omission of either

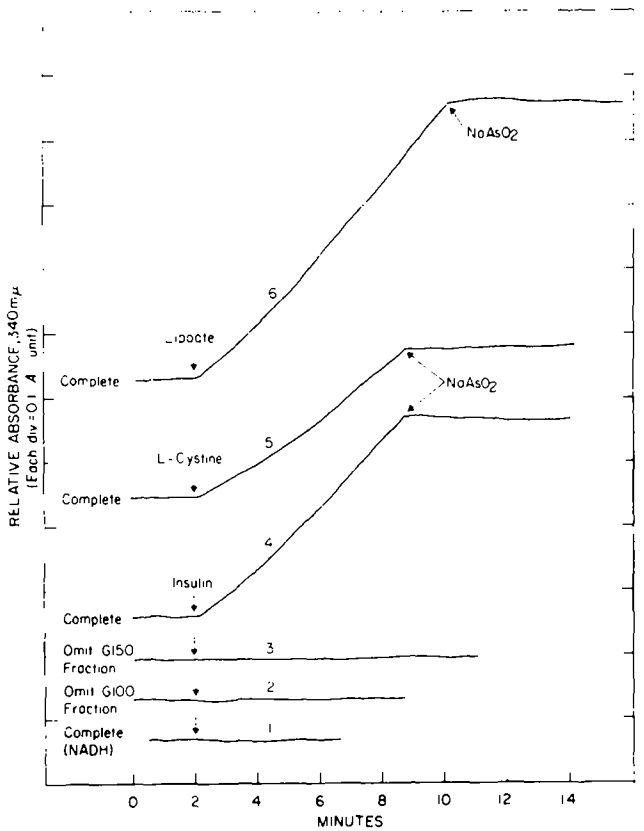

Fig. 7. NADPH-dependent reduction of disulfide substrates by chromatographically purified components of rat liver microsomal supernatant. Reduction of disulfide substrates was measured spectrophotometrically at 340 mμ in 1.0 ml Tris-EDTA (pH 7.5). The Sephadex G-150 and Sephadex G-100 fractions refer, respectively, to the isolated components of Figs. 5 and 6 active in nucleotide-dependent disulfide reduction. Components of the complete systems at zero time were: 0.2 μmole NADPH, 184 μg Sephadex G-150 fraction and 157 μg Sephadex G-100 fraction. Cystine (approx. 0.5 μmole), lipoate (1.0 μmole), insulin (2.0 mg), and arsenite (0.1 μmole) were added at the times indicated. NADH was substituted for NADPH in reaction mixture 1. Cuvettes were balanced with respect to all components except the disulfide substrates.

Sephadex fraction from the insulin-reducing system (Curves 2 and 3) also abolished nucleotide oxidation, as did the substitution of NADH for NADPH in the complete system (Curve 1).

In order to confirm the reducing activities of the foregoing chromatographic components, the formation of insulin sulfhydryl groups in similar mixtures was measured. Incubation mixtures containing insulin, reduced nucleotide, and the Sephadex components were incubated aerobically, and the net thiol formation measured at intervals of time with DTNB in 5 M guanidine, as described earlier[1]. The data, summarized in Fig. 8, confirm the spectrophotometric experiments of the preceding figure and indicate that in the complete system containing NADPH, the extent of reduction of insulin attained 85% of the calculated maximum. As in the case shown in Fig. 7 omission of either Sephadex fraction essentially abolished sulfhydryl for-

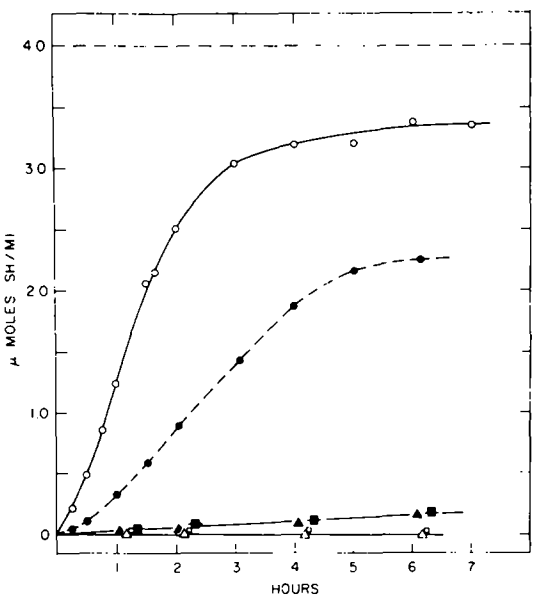

Fig. 8. Reduction of insulin as measured by formation of sulfhydryl groups in reaction mixtures containing reduced nucleotide and chromatographically purified components of rat liver microsomal supernatant. Reaction mixtures were incubated aerobically in stoppered tubes at 25°. At intervals of time aliquots were assayed for sulfhydryl groups with DTNB in 5 M guanidine as described previously[1]. Values shown are corrected for initial sulfhydryl content. Complete reaction mixtures contained, per ml of Tris–EDTA (pH 7.5) the following components: 3.0 μmoles NADPH, 4.0 mg insulin, 184 μg Sephadex G-150 fraction (Fig. 5) and 157 μg Sephadex G-100 fraction (Fig. 6). ○– ○, complete reaction mixture; △ △, complete *minus* Sephadex G-150 fraction; ▲—▲, complete *minus* Sephadex G-100 fraction; □—□, complete *minus* insulin; ■– ■, complete, with NADH in place of NADPH; ●– – – –●, data taken from an earlier experiment in which insulin was incubated with NADPH and 600 μg of the Sephadex G-150 component and no Sephadex G-100 component. The horizontal dashed line at 4.0 μmoles sulfhydryl represents 100% insulin reduction.

mation, as did the substitution of NADH for NADPH. The dashed curve of Fig. 8 shows the results of an earlier experiment, in which insulin reduction was found to occur in a reaction mixture lacking the Sephadex G-100 component but containing a substantially greater quantity of the Sephadex G-150 fraction (600 μg) than that utilized in the other mixtures indicated in this figure (184 μg). The somewhat slower but nevertheless substantial formation of sulfhydryl groups observed in the earlier experiment is compatible with the suggestion that Sephadex G-150 purified heat-labile component is contaminated with a small amount of the heat-stable component.

DISCUSSION

The observation that the nucleotide-dependent disulfide-reducing activity of rat liver cytoplasm is resolvable into heat-labile and heat-stable components, both of which are required for enzyme activity, is consistent with a previous suggestion[1] that such activity may be catalyzed by a thioredoxin or thioredoxin-like system. By analogy with the known heat stabilities of the components of the thioredoxin systems

of *Escherichia coli*[2-4] and rat hepatoma[5-7], it is possible to speculate further that the heat-stable and heat-labile fractions isolated in the present work may correspond, respectively, to thioredoxin and thioredoxin reductase. However, a final conclusion on this point must await further purification and characterization of these isolated products. In particular, it would be of interest to determine whether these fractions can participate in nucleotide-dependent reduction of ribonucleotides by ribonucleotide reductase of tumor or other proliferating tissue.

Inasmuch as the thioredoxin system was originally isolated as a component of a bacterial system participating in ribonucleoside phosphate reduction[2,3] its role in relation to this function has quite naturally been emphasized. However, in view of the presence of essentially identical disulfide-reducing systems in yeast[8,23] which participate in the reduction of L-methionine sulfoxide[9] or activated sulfate[10,11], as well as the observation that non-proliferating liver cells, which lack the ability to reduce ribonucleoside phosphates[5,12,13], contain normal amounts of the thioredoxin system[12], it has been suggested[8] that the thioredoxin system may have a broader function in disulfide reduction.

Although the nature of the heat-stable component is presently unknown, its apparent retention following efficient dialysis for 12–24 h would suggest that it possesses a molecular weight of at least several thousand. In addition, it has been observed that following several cycles of freeze–thaw or prolonged storage at −20°, the cofactor activity of the chromatographically isolated heat-stable component is greatly reduced; however, substantial recovery of activity could be obtained by incubation of the inactive material with 50 mM dithiothreitol, suggesting a requirement for one or more functional sulfhydryl groups.

Recently ONDARZA AND ABNEY[14] have reported the partial purification of a flavin-containing NADPH-dependent CoASSG reductase from the soluble fraction of rat liver. Although the enzyme was similar to the reductase reported here with respect to its inhibition by arsenite, neither its activity toward other disulfide substrates nor its dependence on a heat-stable fraction was reported.

Like the activity exhibited by crude liver extracts[1] the GSH disulfide transhydrogenase activity appearing with the fraction of protein unretained by DEAE-cellulose (Fig. 2) possessed catalytic activity toward both protein (insulin) and non-protein (hydroxyethyldisulfide) substrates. The chromatographically isolated enzyme proved to be very unstable; solutions of the enzyme rapidly acquired a pronounced viscosity followed eventually by precipitation of material and considerable loss of activity. This tendency toward aggregation could account for the rather diffuse distribution of transhydrogenase activity observed both throughout the $(NH_4)_2SO_4$ fractions of rat liver supernatant and in the fraction of resin-adsorbed protein eluted from DEAE-cellulose by the salt gradient (Fig. 2).

The transhydrogenase studied here resembles a number of similar enzymes previously purified from yeast[15], bovine kidney[16], and rat liver[17,18], particularly with respect to its instability and behavior on DEAE-cellulose. In addition, the purified enzyme appeared able to catalyze reduction of disulfide bonds in proteins such as insulin (Fig. 3) and γ-globulin (unpublished). However, these latter reductions required the use of considerably higher concentrations of GSH (*e.g.* 2–5 μmoles/ml) than those generally employed with the simpler non-protein disulfide substrates (*e.g.* 0.1–0.25 μmole/ml), as well as somewhat more alkaline conditions, so that differences

in substrate specificity may possibly be accounted for in terms of the experimental conditions employed.

It is also worthwhile to consider the possible relationship between the transhydrogenase activity described here and the previously described "GSH–insulin transhydrogenase" of bovine liver, whose activity was first reported by KATZEN AND STETTEN[19]. Although this enzyme can also catalyze the reduction of insulin by GSH, as measured by means of the same coupled system, it has considerably less activity toward other disulfide substrates, either protein or non-protein in nature. This difference, as well as the finding (F. TIETZE, unpublished) that rat liver "GSH–insulin transhydrogenase" is most likely microsomal in origin, would indicate that these two enzymes are distinct entities.

The occurrence of two different enzyme systems in liver cytoplasm, each of which appears able to catalyze non-specific reduction of disulfide bonds, raises the question of their intracellular roles. Reference has already been made to the possible identity of the nucleotide-dependent reductase with the thioredoxin system. Speculation as to the specific function(s) of the observed transhydrogenase activity seems unwarranted at the present time other than to note that the presence of this activity in the same soluble fraction which contains also the bulk of the glutathione reductase activity accords logically with the central role generally thought to be played by the glutathione–glutathione reductase system in the maintenance of cellular thiol groups. Possibly these reductive systems are related in some way to recent findings[20,21] that a substantial fraction of cellular thiols of low molecular weight, such as GSH and cysteine, are bound to cytoplasmic proteins in the form of mixed disulfides; regeneration of the component thiols from these mixed disulfides might well involve participation of one or the other of the enzyme systems described here. The possible role of the thiol disulfide transhydrogenase reaction in the reduction of cystine by yeast *in vivo* has been suggested by NAGAI AND BLACK[15]. More recently, STATES AND SEGAL[22] have reported the presence of GSH-cystine transhydrogenase activities in both soluble and lysosomal fractions of rat intestinal mucosa and have commented on the possible bearing of this finding on the deposition of cystine crystals seen in human cystinosis.

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