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GLUTATHIONE REDUCTASE IN THE SEA URCHIN EGG

I. PURIFICATION AND GENERAL PROPERTIES

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SUMMARY

Glutathione reductase (NADPH:GSSG oxidoreductase, EC 1.6.4.2) was purified 6400-fold from sea urchin eggs (*Hemicentrotus pulcherrimus*). The purified preparation was homogeneous on sodium dodecyl sulfate disc electrophoresis. The molecular weights determined by electrophoresis and calculated from the flavin content were 52 000 ± 1000 and 52 300, respectively. The molecular weight of 102 000 ± 4000 of the whole enzyme was determined by gel filtration on Sephadex G-200. The pH optimum was 7.2 and the optimum of potassium phosphate concentration was 0.1 M. The apparent K_m value for GSSG was 0.10 mM and that for NADPH was 5 μM. The apparent K_m for FAD binding to the apoenzyme was 0.78 μM.

INTRODUCTION

Glutathione reductase (NADPH:GSSG oxidoreductase, EC 1.6.4.2) is widely distributed in nature. The enzyme has been extensively purified from *Escherichia coli* [1], pea seedlings [2], rat liver [3], human erythrocytes [4–6], yeast [7–10], Ehrlich ascites tumor cells [11] and *Penicillium chrysogenum* [12] but not from the sea urchin egg or any other Echinodermata.

It has been demonstrated that the amounts of GSH and GSSG are stationary during early cleavage cycle of the sea urchin embryo [13, 14]. The ratio of GSH to GSSG is consistently about 25:1 [14]. A strong activity of glutathione reductase in embryonic cells likely supports the constant ratio.

Recently, Fredborg and Lindahl [15] reported that glutathione reductase activity in Ehrlich ascites tumor cells decreases significantly in mitosis. The aim of our investigations is to clarify the functions of the enzyme in cell cycle of the sea urchin embryo. The experiments described in the present paper were designed to purify and characterize glutathione reductase from the sea urchin egg.

MATERIALS AND METHODS

Biological materials

The embryos of the sea urchin, *Hemicentrotus pulcherrimus* were used as sour-

ces of the enzyme. In some experiments, *Pseudocentrotus depressus* was also used. Gametes were obtained by 0.5 M KCl-induced spawning or by injection of 0.1 M acetylcholine into the body cavity of the animals [16].

Chemicals

NADPH, NAD^+, GSH, FAD, FMN, riboflavin, dithiodipropanol (bis(γ-hydroxypropyl)disulfide) and L-cystine were obtained from commercial sources. DEAE cellulose (1.05 mequiv/g) was purchased from Brown Co. and Sephadex G-200 from Pharmacia. Hydroxylapatite was prepared according to the method of Tiselius et al. [17]. GSSG was prepared by the oxidation of GSH with H_2O_2 under the following two procedures: (1) GSH solution containing equivalent H_2O_2 was aerated for a day at room temperature. (2) GSH solution containing equivalent H_2O_2 and 10^{-5} M $FeCl_3$ was aerated for 30 min, followed by addition of EDTA to a final concentration of 1 mM. GSSG formed was precipitated with acetone and collected by centrifugation. The precipitate was dissolved and recrystallized in 50% ethanol at −20 °C, and was dried in vacuo. This preparation corresponds to $GSSG \cdot 2C_2H_2OH$ [18]. Both preparations were negative for the Ellman reaction [19]. The K_m values for both GSSG preparations were not different at all. NADH was prepared by the reduction of NAD^+ with alcohol dehydrogenase by the method of Racker [20].

Assay of enzymic activity

For the routine assay, the rate of NADPH oxidation was followed by measuring the decrease in absorption at 340 nm ($\Delta A_{340\,nm}$) with a spectrophotometer (Hitachi 139) at 25 °C. The assay mixture contained EDTA (1 mM), GSSG (0.8 mM), NADPH (0.1 mM) and the enzyme in a final volume of 3 ml of 0.1 M potassium phosphate buffer (pH 7.2). A unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μM NADPH per min under the above condition. The specific activity was defined as units per mg protein. During purification of the enzyme, two different procedures for protein assay were employed: (1) the method of Lowry et al. [21] with bovine serum albumin (Iwaikagaku) as a standard; (2) absorbance at 280 nm using an $E^{1\%}_{1cm}$ at 280 nm of 18.6 [9, 12].

Dodecylsulfate gel electrophoresis

The electrophoresis on polyacrylamide gel was carried out according to the method of Weber and Osborn [22] with slight modifications. The purified enzyme was dialysed overnight against a solution of 8 M urea, 1% mercaptoethanol. 0.5% sodium dodecylsulfate, 5 mM EDTA and 20 mM Tris–HCl buffer (pH 8.5). The sample was subsequently alkylated by dialysis against 10 mM *N*-ethylmaleimide, containing 8 M urea, 0.5% sodium dodecylsulfate, 5 mM EDTA and 20 mM Tris–HCl (pH 6.5) for 3 h and again dialysed against the former buffer to remove excess *N*-ethylmaleimide prior to the application on the gel. The gel consisted of 7.5% acrylamide, 0.2% *N,N'*-methylene-bis-acrylamide, 0.01 mM riboflavin, 5% glycerin, 0.05% sodium dodecylsulfate and 25 mM Tris–glycine buffer (pH 8.5).

Molecular weight determination

The molecular weight was determined by the method of Weber and Osborn [22] and that of Andrews [23].

RESULTS

Purification of glutathione reductase

(*1*) *Extraction.* Fertilized eggs of *Hemicentrotus* were collected by low speed centrifugation. After washing with cold water, packed eggs were homogenized in 10 vol. of cold water with a Teflon homogenizer employing 5 to 7 strokes. The homogenate was centrifuged at 10 000 × *g* for 15 min. The supernatant contained above 90% of glutathione reductase in eggs.

(*2*) *Ammonium sulfate fractionation.* The water soluble fraction was brought to 40% saturation with respect to $(NH_4)_2SO_4$ (243 g/l). After centrifuging at 10 000 × *g* for 15 min, the supernatant was brought to 80% saturation (an additional 285 g/l). The solution was stirred for 4 to 6 h and the floating material was collected by centrifuging at 10 000 × *g* for 15 min. When necessary, this floating material was stored as the 80% saturated $(NH_4)_2SO_4$ suspension at 0 °C for the next step. The collected floating material was dialysed against two changes of 20 vol. of 0.05 M phosphate buffer (pH 7.2) containing 1 mM EDTA.

(*3*) *Acetone fractionation.* The dialysed enzyme preparation was brought to −3 °C by stirring in an ice–salt bath. Cold acetone was added drop by drop. A protein fraction precipitating between 33 and 50% acetone (v/v) was collected and suspended in 10 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA. The insoluble material was centrifuged off at 30 000 × *g* for 60 min. The supernatant was dialysed overnight against 100 vol. of the same buffer.

(*4*) *DEAE-cellulose chromatography.* The dialysed supernatant was applied to a DEAE-cellulose column (2.5 cm × 30 cm) and eluted with a linear gradient of KCl (0–0.3 M). Glutathione reductase was eluted with 0.1 M KCl. Those fractions with a high activity were combined and dialysed overnight against 1 l of 100% saturated $(NH_4)_2SO_4$ solution containing 1 mM EDTA at pH 7.2. The protein precipitating during dialysis was collected by centrifugation at 10 000 × *g* for 15 min and dissolved in 7 ml of 10 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA.

(*5*) *Sephadex G-200 chromatography.* The above solution was applied to a Sephadex G-200 column (3 cm × 60 cm) equilibrated with 10 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA and eluted with the same buffer. Those fractions with a high activity were combined and concentrated to 5 ml by ultrafiltration (Dia Flo XM 50, Amicon Co.).

(*6*) *DEAE cellulose rechromatography.* The concentrated solution was applied to a DEAE-cellulose column (1.1 cm × 12 cm) equilibrated with 0.05 M KCl, 0.1 mM EDTA, 10 mM phosphate buffer (pH 7.7) and eluated with a linear gradient of KCl (0.05–0.20 M). Those fractions with a high activity (Fig. 1A) were combined and dialysed overnight against 100% saturated $(NH_4)_2SO_4$. The precipitate was collected, dissolved in 1 ml of 10 mM phosphate buffer (pH 7.5) and dialysed against two changes of 500 vol. of the same buffer.

(*7*) *Hydroxylapatite chromatography.* The dialysed enzyme preparation was applied to a hydroxylapatite column (1.1 cm × 9 cm) equilibrated with 10 mM phosphate buffer (pH 7.5) and eluated with a linear gradient of phosphate buffer (0.01–0.06 M). Those fractions with a high activity (Fig. 1B) were collected and concentrated to 2 ml by ultrafiltration.

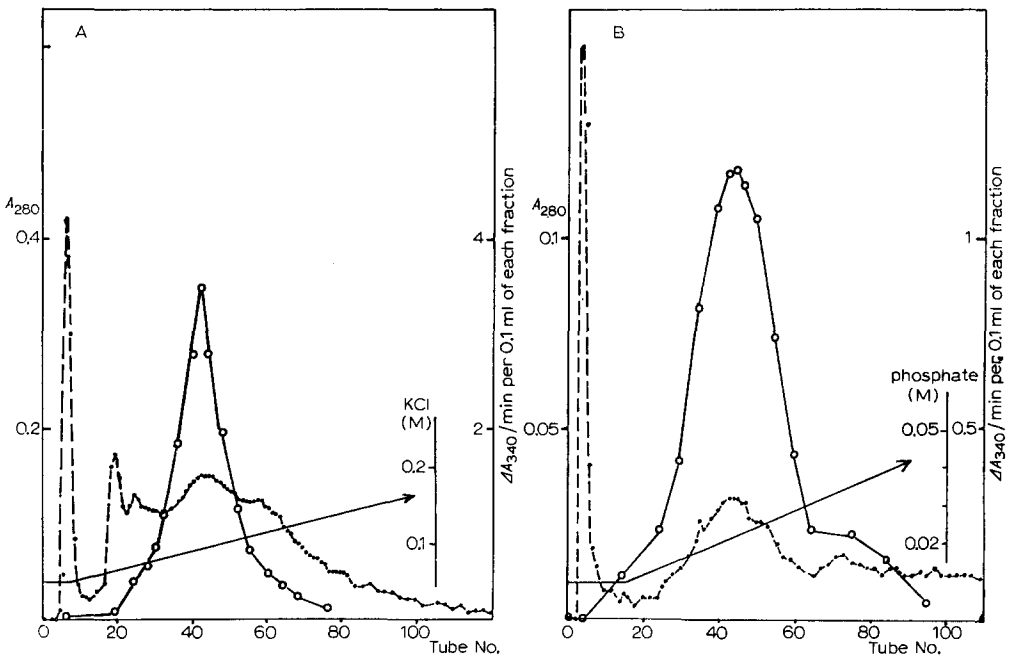


Fig. 1. (A) Rechromatogram on DEAE-cellulose of an enzyme fraction fractionated by Sephadex G-200 chromatography. 5 ml of the enzyme fraction obtained in Step 5 (17 mg protein) was applied to a DEAE-cellulose column. Each fraction of 2.9 ml was analysed for protein and enzymic activity. (B) Column chromatogram on hydroxylapatite of an enzyme fraction obtained by DEAE-cellulose rechromatography. 1 ml of the enzyme fraction obtained in Step 6 (4.3 mg protein) was subjected to a linear gradient elution through a hydroxylapatite column. Each fraction of 2.5 ml was analysed for protein and enzymic activity. ●---●, absorbance at 280 nm; ○—○, glutathione reductase activity.

All operations except acetone fractionation were carried out at 0–4 °C.
The data from a typical purification procedure are summarized in Table I.

Electrophoresis of purified enzyme

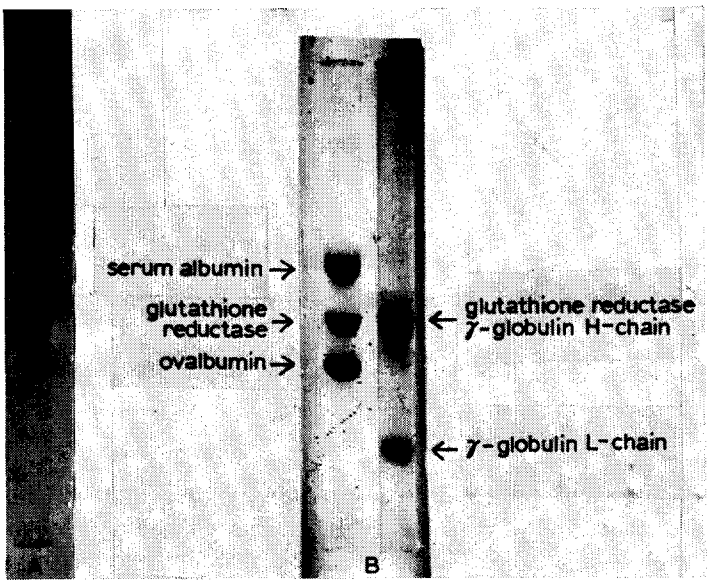
The electrophoresis on polyacrylamide gel was carried out to check the purity

TABLE I

PURIFICATION OF SEA URCHIN GLUTATHIONE REDUCTASE

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification factor
1 Crude extract	40 000	2000	0.05	100	1.0
2 40–80% $(NH_4)_2SO_4$ fraction	13 000	1800	0.14	90	2.8
3 33–50% acetone fraction	3 800	1700	0.45	85	9.0
4 DEAE-cellulose, 1st	52	1000	19	50	380
5 Sephadex G-200	17	900	53	45	1100
6 DEAE-cellulose, 2nd	4.3	630	145	32	2900
7 Hydroxylapatite	1.0	330	320	17	6400

of purified enzyme and to determine the molecular weight of its subunit using the method of Weber and Osborn [22]. The purified enzyme exhibited one band on dodecylsulfate disc electrophoresis (Fig. 2A). From Figs 2B and 2C, the molecular weight of the subunit was calculated to be 52 000 $\pm$ 1000.



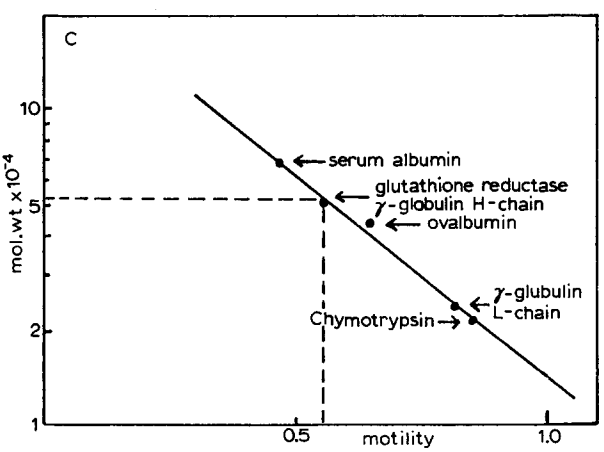


Fig. 2. (A) Electrophoresis of purified enzyme on dodecylsulfate polyacrylamide gel. About 40 μg protein was applied on the gel. (B) and (C) Determination of the molecular weight of sea urchin glutathione reductase by dodecylsulfate polyacrylamide gel electrophoresis. Bovine serum albumin (Iwaikagaku), ovalbumin (Sigma), γ-globulin H- and L-chains (Armour), and chymotrypsin (Sigma) were used as marker proteins. Glutathione reductase and H-chain of γ-globulin could not be separated as shown in Fig. 2B, right gel. Electrophoresis was carried out on 7.5% polyacrylamide gel (0.4 cm $\times$ 7 cm) at 1 mA and 100 V for about 1.5 h. Bromphenol blue was used as tracking dye. The gel was stained by coomasie brilliant blue in 40% methanol and 10% acetic acid overnight and destained by several changes of 7% acetic acid.

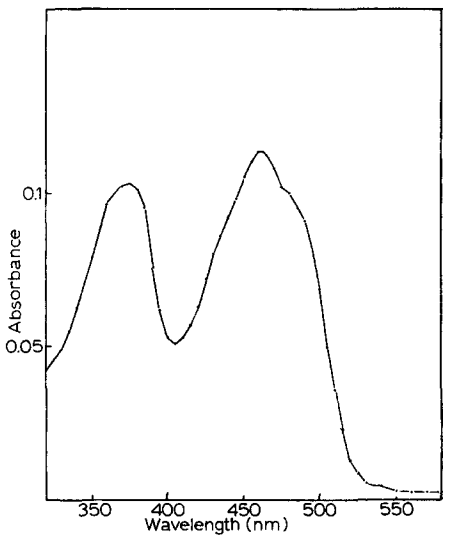


Fig. 3. Absorbance spectrum of purified glutathione reductase (0.53 mg enzyme/ml of 20 mM potassium phosphate buffer (pH 7.5)).

Absorbance spectrum

The concentrated purest enzyme solution was yellow colored and had an absorption spectrum characteristic for a flavoprotein (Fig. 3). The maxima revealed at 275, 375 and 460 nm. The absorbance ratios for the different maxima were $A_{280\text{nm}}/A_{460\text{nm}} = 8.6$ and $A_{375\text{nm}}/A_{460\text{nm}} = 0.91$. In Table II, values are given for the purified

TABLE II

REFERENCE VALUES OF THE ABSORPTION SPECTRUM AND THE MOLECULAR WEIGHT OF GLUTATHIONE REDUCTASE

Source	$\lambda_{max.}$ (nm)	$A_{280\text{ nm}}/A_{460\text{ nm}}$	$A_{375\text{ nm}}/A_{460\text{ nm}}$	Mol. wt (min.)	Mol. wt	Reference
Yeast	462		0.91*		118 000	Colman and Black [8]
	372; 462	8.9–9.3	0.95*	56 500		Massey and Williams [9]
	280; 370; 460			56 000	124 000	Mavis and Stellwagen [10]
Erythrocyte	275; 365–375; 460–462	8.8	0.90	65 500	120 000	Icen [5]
	278; 372; 462	9.0	1.1	56 600	115 000	Staal et al. [6]
Penicillium	275; 365; 462	9.0–9.5	0.89*	55 000	110 000	
					109 000	Woodin and Segel [12]
					110 000	
Sea urchin	275; 375; 460	8.6	0.91	52 300	105 000	
				52 000	104 000	this publication
					102 000	

* The values were calculated from the absorption spectra in the references.

enzymes from other sources together with those of the sea urchin. From those data, it can be concluded that the values of the present enzyme are in good agreement with those from other materials. The prosthetic group seemed to be FAD. Based on an $E^{1\%}_{1cm}$ at 280 nm of 18.6 for glutathione reductase and a molar extinction coefficient at 460 nm of $1.13 \cdot 10^4$ for enzyme-bound flavin, a minimum molecular weight of 52 300 per mole FAD could be calculated.

Molecular weight determined by gel filtration

Upon application to Sephadex G-200, the purified enzyme eluted as a single protein peak with constant specific activity. According to the method of Andrews [23], a molecular weight of 102 000 ± 4000 was calculated (Fig. 4).

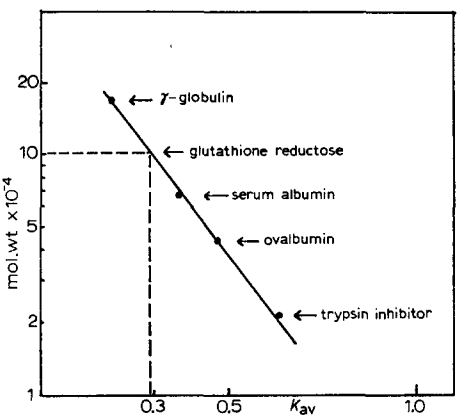


Fig. 4. Determination of molecular weight of purified enzyme by gel filtration on Sephadex G-200. γ-Globulin, serum albumin, ovalbumin and trypsin inhibitor (Sigma) were used as protein markers. The column size was 2.1 cm × 66 cm and elution was performed by 50 mM potassium phosphate (pH 7.2) containing 0.1 mM EDTA.

Other properties of glutathione reductase

The pH optimum was 7.2 to 7.3. In potassium phosphate buffer at pH 7.2, optimum activity was observed at 0.1 M phosphate. Addition of 1 mM EDTA to the assay solution increased the apparent activity about 10%. Further addition of EDTA showed no more effect. The observed activity increased a little with increasing KCl concentration until 0.1 M but it decreased with further increasing KCl concentration.

The apparent K_m values were 0.10 mM for GSSG and 5 μM for NADPH under the present experimental conditions. NADH was less than 1% as active as NADPH as a reductant. L-Cystine and dithiodipropanol were inactive as an oxidant. The enzyme was very stable. No significant loss of activity was detected when reserved at 0 °C in 80% saturated $(NH_4)_2SO_4$ solution for a few months or when stored freezing in 50 mM phosphate buffer at −18 °C for a few months.

Reactivation of the apoenzyme by FAD

The apoenzyme was prepared according to the method of Asnis [1]. The 33–50% acetone fraction (specific activity, 0.5 unit/mg protein) was added to an equal

vol. of saturated $(NH_4)_2SO_4$ solution and brought to pH 1.0 with concentrated HCl. After stirring vigorously for 10 min at 0 °C, the apoenzyme was collected by centrifugation and was suspended in a volume of 0.1 M phosphate buffer (pH 7.2) containing 1 mM EDTA equal to the original. The solution had only 1 % of its original activity.

TABLE III

RECOVERY OF GLUTATHIONE REDUCTASE ACTIVITY OF THE APOENZYME BY FLAVINS

After a 15 min incubation at 25 °C of the apoenzyme in 0.1 M potassium phosphate (pH 7.2) containing 1 mM EDTA with FAD (8.4 μM) or FMN (18 μM) or riboflavin (18 μM) ,0.1 ml of samples was withdrawn and activity was measured.

Enzyme treatment	*Hemicentrotus*		*Pseudocentrotus*	
	$\Delta A_{340\ nm}$ per min	% original activity	$\Delta A_{340\ nm}$ per min	% original activity
Intact enzyme	0.72	100	1.0	100
Apoenzyme	0.010	1.4	0.007	0.7
Apoenzyme plus riboflavin	0.012	1.7	0.010	1.0
Apoenayme plus FMN	0.012	1.7	0.010	1.0
Apoenzyme plus FAD	0.238	33	0.18	18

Riboflavin and FMN could not restore the enzymatic activity and only FAD could restore the activity to 33 % of its original as shown in Table III. Fig. 5 shows the effect of the FAD concentration on the reactivation of the apoenzyme. From these data, the concentration of FAD required to give half maximal activity was 0.78 μM. The same experiment with the apoenzyme of glutathione reductase from *Pseudocentrotus depressus* gave a value of 0.81 μM.

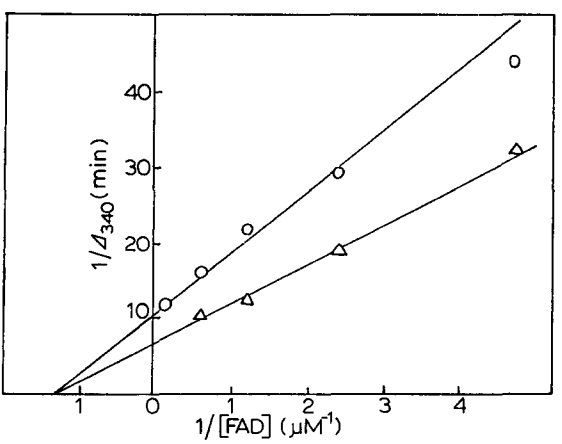


Fig. 5. Effect of FAD concentration on recovery of glutathione reductase activity of the apoenzymes. After a 15 min incubation of the apoenzymes with various concentrations of FAD as indicated, activities were measured. △, *Hemicentrotus*; ○, *Pseudocentrotus*.

DISCUSSION

The sea urchin glutathione reductase purified 6400-fold from *Hemicentrotus pulcherrimus* is highly pure, judging from its behavior on gel-electrophoresis, its elution pattern from a Sephadex G-200 column, its absorption spectrum and its high specific activity.

The absorption spectrum and the reactivation of the apoenzyme by FAD proved that the prosthetic group of the sea urchin glutathione reductase is FAD, similar to those of glutathione reductases from other sources, *E. coli* [1], pea seedling [2], yeast [9, 10], human erythrocyte [4–6] and *Penicillium chrysogenum* [12].

The molecular weight of glutathione reductase in the sea urchin egg is a little smaller than those of yeast, human erythrocyte and *Penicillium* (see Table II), but enzymic properties are little different from those obtained from the above materials. A minimum molecular weight of 52 300 per mole FAD could be calculated from the flavin content. This value agrees with the value of 52 000 determined by dodecylsulfate disc electrophoresis. Mavis and Stellwagen [10] showed that the yeast enzyme contains two subunits, having a molecular weight of 56 000 and that it is separated into two subunits in 1 % sodium dodecylsuflate. Therefore, the molecular weight of 52 000 determined by dodecylsulfate electrophoresis must be a molecular weight of its subunit. In combination with the molecular weight of 102 000 for the sea urchin glutathione reductase as determined by the method of Andrews [23], it can be concluded that the native enzyme contains two moles of FAD and is built up by two polypeptide chains. This seems to be general for glutathione reductase.

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