Glutathione Reductase from Germinated Peas

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Since the discovery of glutathione reductase in plants (Mapson & Goddard, 1951; Conn & Vennesland, 1951) there has been little work on the nature and properties of the enzyme. In the work already published, there is some difference of opinion as to the nature of the prosthetic group of the enzyme prepared from both plant and animal sources. Langdon (1958) reported that the enzyme extracted from rat liver contained no flavin nucleotide, and Moustafa (1955) reported similarly on the plant enzyme. However, Asnis (1955) and Mapson (1959) both claimed that the enzyme isolated from bacterial and plant sources respectively contained flavin-adenine dinucleotide (FAD) as the prosthetic group.

In the present paper we have described the isolation of the enzyme from peas and, although a pure protein has not as yet been obtained, the degree of purification achieved has been sufficient to warrant a more detailed study of the nature and properties of the enzyme. We have confirmed that the enzyme is a flavoprotein and the present paper records our observations on some of its properties.

EXPERIMENTAL

Chemicals

NADP⁺, NAD⁺, GSH, GSSG and the thiol reagents *N*-ethylmaleimide and *p*-chloromercuribenzoate were purchased from L. Light and Co. Ltd., Colnbrook, Bucks. NADPH was prepared by the reduction of NADP⁺ with isocitrate in the presence of isocitrate dehydrogenase by the method of Evans & Nason (1953), and NADH was prepared by the reduction of NAD with alcohol dehydrogenase by the method of Racker (1950). FAD was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and triethylaminoethylcellulose (0.64 m-equiv. of basic nitrogen/g.) from Serva, Heidelberg, Germany. Glass-distilled water was used throughout and buffer salts were treated with 8-hydroxyquinoline before use to remove heavy metals. The Visking tubing used in our experiments was pretreated to remove thiol components in one of the following ways: either the tubing was soaked and washed with 0.1 *N*-H₂SO₄ until no further thiol components were detectable (Liddell & Saville, 1959) and then finally washed in water, or, in experiments in which *p*-chloromercuribenzoate was used, the tubing was soaked in a solution of mM-*p*-chloromercuribenzoate and washed with water before use.

Assay methods

Assay of enzymic activity. The rate of oxidation of NADPH was followed by measuring the decrease in

absorption at 340 mμ in a Hilger Uvispek spectrophotometer at 20°. A final volume of 3 ml. of 0.1 *M*-Na₂HPO₄-KH₂PO₄ buffer (1 mM with respect to EDTA) contained the enzyme, GSSG (0.8 mM) and NADPH (0.1 mM). Under these conditions the rate of reaction was proportional to the enzyme concentration and was linear with time. A unit of enzyme activity was defined as the amount of enzyme that catalysed the reduction of 1 μmole of oxidized glutathione in 1 min. at 20°. For a detailed study of the kinetics of the reaction, the rate of oxidation of the NADPH was followed with a recording fluorimeter (Dalziel, 1962).

Estimation of flavin. The FAD content of the enzyme was determined by the D-amino acid-oxidase test (Huennekens & Felton, 1957), after separation of the flavin from the apoenzyme either by (a) heating for 5 min. at 100°, or (b) proteolytic digestion with trypsin and chymotrypsin in 0.05 *M*-pyrophosphate buffer, pH 7.8, for 4 hr. at 37° (Kearney, 1960). As we had evidence that the flavin was more easily split from the apoenzyme when the enzyme itself was in the reduced condition, assays of the flavin content were repeated after the enzyme had been reduced by NADPH. We found that the same amount of flavin was obtained whether the enzyme was in the oxidized or the reduced state, though splitting by heat gave slightly higher values (10%) than those obtained after enzymic digestion. The mean of all four types of experiments showed that 1 μmole of FAD was released from an amount of enzyme equivalent to 3800 units of activity, individual assays giving a value within ±10% of this value.

We have as yet been unsuccessful in obtaining the enzyme as a pure protein, for sedimentation analysis in the Spinco analytical ultracentrifuge showed that our best preparations were still contaminated with a small amount of foreign protein. Because of this, we have calculated the concentration of enzyme in the different experiments on the basis of the amount of flavin present, on the assumption that 1 mole of enzyme contains 1 mole of FAD. This assumption is based on the fact that the enzyme has a molecular weight of approximately 60 000, calculated from our sedimentation data, and this amount of enzyme we have found contains almost exactly 1 mole of flavin.

Estimation of protein, glutathione and nicotinamide-adenine dinucleotide phosphate. The protein content of the various enzyme preparations was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), GSH by the method of Liddell & Saville (1959) and GSSG by the same method after enzymic reduction. NADP⁺ was estimated by measuring the increase of absorption at 340 mμ after reduction by the isocitrate-dehydrogenase system (Evans & Nason, 1953).

Reduction of disulphides by potassium cyanide. Conditions for the reduction of disulphide bonds with KCN were determined with GSSG. With 100 mM-KCN at pH 7.6 the reaction with GSSG was completed in a few minutes, when

60–70% of the disulphide was reduced; with 10 mM-KCN, 15 min. was necessary for the completion of the reaction (40–50% of the disulphide regenerated as GSH). At lower concentrations, no measurable reduction of GSSG occurred within 1.5 hr. at room temperature. We therefore employed KCN at concentrations of 10–100 mM in the enzyme experiments described in the text.

Separation of products of reaction from enzyme. Separation of the GSH or NADP⁺ or both, formed from the reduced enzyme on the addition of GSSG, and from the oxidized enzyme on the addition of NADPH, were carried out by filtration of the solutions under reduced pressure through collodion shells (special membrane filters impermeable to proteins) purchased from Membranfilter Gesellschaft, Göttingen, Germany. Oxidation was minimized by keeping the solutions under nitrogen during the filtration. A solution of the enzyme in a total volume of 3 ml. of 0.01 M-phosphate buffer, pH 7.4 (1 mM with respect to EDTA), after the addition of either GSSG or NADPH, was placed in the collodion shell, which was then immersed in 8 ml. of water (1 mM with respect to EDTA) in an outer vessel; in the assay for NADP⁺, the EDTA was omitted. Filtration was continued until about 0.1 ml. was left in the shell, the diffusible components being collected in the outer vessel. The procedure, which took approximately 2 hr. to complete, was repeated three times, the total filtrates being combined and where necessary concentrated by freeze-drying. To check the efficiency of the filtration process, the fall in concentration of a solution of GSH (80 µg./ml.) put through the same procedure was measured: the amount of GSH left in the collodion shell was then too small to be detected (less than 0.5 µg.).

Measurement of radioactivity. Samples (0.05 ml.) of the solutions to be examined were pipetted on to circles of paper on aluminium planchets and allowed to dry. The radioactivity was determined with a Geiger counter. The apparatus consisted of an Ekco scaler N 529 B, sample oven N 619 and GM tube holder N 620. Counting of each sample continued for sufficient time to give a minimum count of 10 000. The background count, determined in several experiments, was 10.7 counts/min. and in the results this has been subtracted from the total counts; the counting efficiency was 1.56%.

Preparation of the enzyme

Step 1: preparation of extract. 'Laxton Superb' peas were soaked for 12 hr. in tap water and then germinated on wet cotton wool for 3 days at 20°. The pea seedlings (1 kg.) were ground in an end-runner mill with 1 l. of chilled 0.1 M-phosphate buffer, pH 7.4 (1 mM with respect to EDTA), and a trace of a silicone antifoaming agent. In the subsequent steps in the purification EDTA was maintained at a concentration of 1 mM in all solutions. The extract was pressed through muslin and the pH adjusted to 5.5 by the addition of 3 N-HCl. After centrifuging at 20 000g for 15 min. the clear supernatant was adjusted to pH 6.9 by the addition of 3 N-NaOH.

Step 2: fractionation with ammonium sulphate. To the cooled (3°) supernatant (18.4 g. of protein in 1 l.) was added solid (NH₄)₂SO₄ (17.6 g./100 ml. of soln.) to give 30% saturation. The precipitate was centrifuged and discarded. To the supernatant was added further solid (NH₄)₂SO₄ (19.8 g./100 ml. of soln.) to give 60% saturation; the

mixture was centrifuged and the precipitate dissolved in 100 ml. of 0.01 M-phosphate buffer, pH 6.8.

Step 3: heat inactivation. The solution was dialysed overnight against 1 l. of 0.01 M-phosphate buffer, pH 6.8, and then heated rapidly with stirring to 60° in a water bath and maintained at that temperature for 1 min. After rapid cooling to 10° the precipitate was centrifuged down and discarded.

Step 4: fractionation with acetone. The clear supernatant was cooled to -3° in a brine bath kept at -3° and then chilled acetone was added drop by drop down the side of the flask until a concentration of 34% (v/v) was reached. After the mixture had been kept in the cold for 10 min. the precipitate was removed by centrifuging at 20 000g for 5 min. and discarded. Further acetone was now added to the supernatant at -3° to bring the concentration up to 48% (v/v). After 10 min. the precipitate that formed was centrifuged down, dried *in vacuo* and finally stirred into 40 ml. of 0.01 M-phosphate buffer, pH 7.4. The insoluble material was centrifuged off and the clear yellow supernatant dialysed for 4 hr. against 1 l. of the same buffer.

Step 5: fractionation with ammonium sulphate. To the cooled (3°) solution (1.3 g. of protein in 40 ml.) was added solid (NH₄)₂SO₄ (24.3 g./100 ml. of soln.) to give 40% saturation, and the precipitate which formed was centrifuged off and discarded. To the supernatant was added further solid (NH₄)₂SO₄ (13.2 g./100 ml. of soln.) to give 60% saturation; the mixture was centrifuged and the precipitate dissolved in 30 ml. of 0.01 M-phosphate buffer, pH 6.8. This solution was dialysed against 1 l. of the same buffer overnight.

Step 6: adsorption on calcium phosphate gel. The solution (0.79 g. of protein in 30 ml.) was treated with Ca₃(PO₄)₂ gel (1 mg. of gel/mg. of protein) prepared according to Keilin & Hartree (1938), left for 10 min. in the cold and centrifuged. To elute the enzyme the gel was treated successively with 20 ml. of 0.05 M-phosphate buffer, pH 7.8, for 10 min. and, after centrifuging, with 20 ml. of 0.1 M-phosphate buffer, pH 7.4, also for 10 min. The two eluates were combined and dialysed against 1 l. of 0.1 M-phosphate buffer, pH 7.4.

Step 7: fractionation with ammonium sulphate. To the cooled (3°) supernatant (0.4 g. of protein in 40 ml.) was added solid (NH₄)₂SO₄ (32.8 g./100 ml. of soln.) to give 52% saturation. After 10 min. the mixture was centrifuged and the precipitate dissolved in 15 ml. of 0.01 M-phosphate buffer, pH 7.4, and dialysed overnight against 1 l. of the same buffer.

Step 8: adsorption on triethylaminoethylcellulose. This was carried out in a cold room at 5°. Before use the triethylaminoethylcellulose (TEAE-cellulose) was suspended in 0.01 M-phosphate buffer, pH 7.4, and centrifuged; the process was repeated several times. The purified TEAE-cellulose (12 mg. of adsorbent/mg. of protein) was poured into the chromatography column as a thick slurry and allowed to pack down under a head of 10 cm. Hg pressure. The enzyme solution (0.31 g. of protein in 15 ml.) was then added to the column without pressure and the column washed well with 0.01 M-phosphate buffer, pH 7.4, until all protein not adsorbed had been removed. The adsorbed enzyme was eluted with 0.08 M-phosphate buffer, pH 7.4, the progress of the enzyme being followed by the movement down the column of a wide yellow band. Fractions

(10 ml.) were collected and examined and those containing enzyme activity were combined. To the combined eluates was added solid $(\text{NH}_4)_2\text{SO}_4$ (47.2 g./100 ml. of soln.) to give 70% saturation, and after 10 min. the mixture was centrifuged. The precipitate was dissolved in 10 ml. of 0.01 M-phosphate buffer, pH 7.4, and dialysed against 1 l. of the same buffer overnight.

Step 9: gradient elution from triethylaminoethylcellulose. The preparation of the column and the application of the enzyme (0.06 g. of protein in 10 ml.) were carried out as for step 8, the ratio of adsorbent to protein being increased to 120:1. The adsorbed enzyme was eluted by developing the column with 0.01 M-phosphate buffer, pH 7.4, which contained progressively increasing amounts of NaCl. The salt-gradient buffer was prepared by dripping 0.3 M-NaCl in 0.01 M-phosphate buffer, pH 7.4, into 150 ml. of the phosphate buffer alone and continuously feeding the overflow to the column. Samples (3 ml.) were collected from the column and those with a high specific activity (units of enzyme activity/mg. of protein) combined. The enzyme was precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to give 70% saturation, dissolved in buffer and dialysed as in step 8.

The enzyme was further purified by repeating step 9 several times, with the ratio of adsorbent to protein kept at 100:1. The final solution of enzyme in 0.01 M-phosphate buffer, pH 7.4, was concentrated to 5 ml. by filtration through a collodion shell (see the 'Separation of products of reaction from enzyme' section above) and then kept at -3° ; no loss of activity was observed over periods of up to 1 year.

The purification achieved by these operations is summarized in Table 1.

RESULTS

Kinetics of the reaction

Mechanism. The rate of the enzyme reaction, under conditions in which the concentrations of both GSSG and NADPH were varied, was determined with a recording fluorimeter (Dalziel, 1962). With this instrument very small changes in the

concentration of NADPH can be measured. The initial rates of reaction with a range of concentration of GSSG in the presence of different amounts of NADPH were plotted by the Lineweaver & Burk (1934) method (Fig. 1a). The intercepts of the primary plots were replotted against the different concentrations of NADPH (Dalziel, 1957). The results (Fig. 1b) so obtained are consistent with the mechanism described by Alberty (1953) for a two-substrate system, in which the enzyme exists in two different states (oxidized and reduced) and where the substrates combine or react with the different forms of the enzyme. The overall reaction can be pictured as occurring in two stages (equations 1 and 2). The relationships between the con-

centrations of the two substrates, GSSG and NADPH, are thus similar to that reported for lipoate dehydrogenase and its substrates (Massey, Gibson & Veeger, 1960) and for D-amino acid oxidase (Massey, Palmer & Bennett, 1961).

The information obtained from the primary Lineweaver & Burk (1934) plots gives the limiting rate at infinite concentration of the first substrate at any particular concentration of the second substrate, and that from the secondary plots of the intercepts against the reciprocals of the different concentrations of the second substrate gives the K_m of the second substrate at infinite concentration of the first substrate, the intercept in this case being equal to $1/V$, V being the limiting rate with infinite concentration of both substrates. Under the conditions of our experiments (enzyme

Table 1. *Stages in the purification of glutathione reductase from germinated peas*

Experimental details are given in the text.

Stage of purification	Total units (units/1000 g. fresh wt. of pea seedlings)	Total protein (mg./1000 g. fresh wt. of pea seedlings)	Specific activity (units/mg. of protein)
Extraction of peas with 0.1 M-phosphate buffer, pH 7.4, followed by acid treatment (step 1)	570	18 400	0.03
30-60% saturation with $(\text{NH}_4)_2\text{SO}_4$ and dialysis (step 2)	480	9 160	0.05
Heat treatment (step 3)	395	5 350	0.075
34-48% saturation with acetone and dialysis (step 4)	369	1 340	0.28
40-60% saturation with $(\text{NH}_4)_2\text{SO}_4$ and dialysis (step 5)	321	792	0.4
Adsorption on $\text{Ca}_3(\text{PO}_4)_2$ gel and elution (step 6)	280	435	0.64
0-52% saturation with $(\text{NH}_4)_2\text{SO}_4$ and dialysis (step 7)	230	315	0.73
Adsorption on TEAE-cellulose and elution (step 8)	195	60	3.3
Adsorption on TEAE-cellulose and gradient elution (step 9)	130	15	9
Final adsorption on TEAE-cellulose and gradient elution	100	1.5	65

in 0.1M-phosphate buffer, pH 7.4, 1 mM with respect to EDTA, at 20°, the K_m for NADPH at infinite concentration of GSSG was 4.7 μ M and the K_m for GSSG at infinite concentration of NADPH 17 μ M, and V (the limiting rate with infinite concentrations of both substrates) was 1.6×10^4 moles of NADPH oxidized/min./mole of flavin.

Fig. 1. (a) Rate of glutathione-reductase reaction as influenced by the concentration of substrates. Assay of enzyme activity was at 20° in 0.1M-phosphate buffer, pH 7.4 (1 mM with respect to EDTA), in a total volume of 4 ml. Concn. of NADPH: ●, 6.75 μ M; ×, 2.7 μ M; △, 1.35 μ M; □, 0.82 μ M; ▲, 0.54 μ M; ○, 0.27 μ M. Reaction was begun by the addition of 0.75 μ mmole of enzyme. (b) Effect of NADPH concentration on the $V(\text{GSSG})$ values obtained for the experiment described in (a). V (the limiting rate with infinite concentrations of both substrates) is 1.6×10^4 moles of NADPH oxidized/min./mole of enzyme.

Equilibrium constant. Previous work on plant glutathione reductase (Mapson & Goddard, 1951; Conn & Vennesland, 1951), in which the increase in absorption at 340 m μ was used to follow the reaction, failed to demonstrate the reverse reaction, namely the formation of NADPH from NADP $^{+}$ and GSH. By using the more sensitive fluorimetric analysis we have been able to show that NADPH is formed and to estimate its concentration, and from this information to calculate the equilibrium constant, K , for the reaction (Table 2). The values of K at pH 7.4 and 8.3 agree well, and also the value of K at pH 9.3, if we take into account that the sulphhydryl group of GSH is approximately 50 % ionized at this pH (pK 9.1) and if it is assumed that only the un-ionized sulphhydryl group of the GSH reacts with the oxidized enzyme. The measurements were made at 20°.

Inhibition by alkylating and mercaptide-forming reagents

Inhibition by *N*-ethylmaleimide. *N*-Ethylmaleimide forms stable compounds with thiols (Hanes, Hird & Isherwood, 1951). If therefore the integrity of a free sulphhydryl group on the active centre of the enzyme is necessary for the reaction, this reagent should be a potent inhibitor. When *N*-ethylmaleimide was added to the enzyme, even at a high concentration (1 mM), and there action initiated by the addition of GSSG and NADPH, only slight inhibition was observed during the first few minutes even if the inhibitor had been in contact with the enzyme for some time (30 min. at 20°) before the substrates were added. However, inhibition of the enzyme increased as the reaction proceeded. With 0.02 mM-*N*-ethylmaleimide only a very slight inhibition was observed during the first 8 min. of the reaction.

When *N*-ethylmaleimide was added together with NADPH to the enzyme and the reaction was initiated by the addition of GSSG, a variable degree of inhibition, even at the start of the

Table 2. *Equilibrium constant, K , for the glutathione-reductase reaction at various pH values*

Experimental details are given in the text. The equilibrium constant is given by:

$$K = \frac{[\text{NADPH}][\text{GSSG}][\text{H}^{+}]}{[\text{NADP}^{+}][\text{GSH}]^2}$$

pH	Initial concn. of NADP $^{+}$ (mM)	Initial concn. of GSH (mM)	Equilibrium concn. of NADPH (μ M)	$10^{13} K$
7.4	0.08	8.3	0.28	5.7
7.4	0.08	25	0.9	6.5
8.3	0.08	25	2.44	6.0
9.3	0.08	25	3.7	1.6

reaction, was observed, depending on the concentration of inhibitor and the time elapsing before GSSG was added. With 0.02 mM-*N*-ethylmaleimide, maximum inhibition resulted only if the inhibitor and NADPH were left with the enzyme for at least 10 min. at 20° before the addition of GSSG (Fig. 2). The results also show that, when the enzyme was treated with either NADPH or *N*-ethylmaleimide and left for 10 min., and then either *N*-ethylmaleimide or NADPH added followed by GSSG and the activity determined, no immediate inhibition was observed; they thus confirm that the time the enzyme is in contact with both NADPH and *N*-ethylmaleimide is the important factor determining the extent of the inhibition. The gradual development of inhibition

that occurred in the presence of NADPH and the lower concentrations of *N*-ethylmaleimide appears to be due to a slow reaction between the enzyme and inhibitor, rather than to a slow reaction between enzyme and reduced coenzyme, for increasing the concentration of *N*-ethylmaleimide, but not of NADPH, increased the rate of development of the inhibition. These results indicate that *N*-ethylmaleimide reacts with some group on the enzyme only after pretreatment with NADPH.

This interpretation is supported by results obtained from experiments in which (a) the enzyme and *N*-ethylmaleimide (0.1 mM) and (b) the enzyme, *N*-ethylmaleimide and NADPH (0.01 mM) were mixed and then left for 30 min. at 20° for any reaction to go to completion. Both solutions were then dialysed for 17 hr. at 1° against frequent changes of 0.1 M-phosphate buffer, pH 7.4 (1 mM with respect to EDTA) to remove excess of *N*-ethylmaleimide or NADPH. On determination of the enzyme activity, no inhibition was observed in the first case, but almost complete inhibition resulted in the second. The inhibition produced by *N*-ethylmaleimide, once established, was irreversible, for neither prolonged dialysis nor treatment with high concentrations (1–10 mM) of GSSG or GSH reversed it. It seems clear that *N*-ethylmaleimide reacts only with the reduced form of the enzyme and that the combination is irreversible. Yet the presence of GSSG in the enzyme digest partially protects the enzyme against inhibition by *N*-ethylmaleimide, as shown by the results given in Fig. 2 and in more detail in Fig. 3. This suggests that *N*-ethylmaleimide and GSSG compete for the same active group on the enzyme. Analysis of the kinetics of the enzyme reaction in the presence and absence of *N*-ethylmaleimide, in particular plotting s/v against s , and v against v/s , where v is the velocity of the reaction and s the substrate (GSSG) concentration, shows that the inhibition is of the mixed competitive type (Dixon & Webb, 1958). The most plausible explanation is that, in an enzyme digest where both NADPH and an excess of GSSG are present, the concentration of the reduced enzyme will depend inversely on the concentration of GSSG, and, since it is the reduced enzyme which reacts with *N*-ethylmaleimide, the rate of inhibition by *N*-ethylmaleimide in such a solution will depend on the concentration of GSSG present. GSSG will in fact appear to protect the enzyme against inhibition because an increased concentration will diminish the reduced enzyme present and therefore the rate of inhibition by *N*-ethylmaleimide. The following observations are therefore of interest. When the enzyme was first treated with NADPH, and then *N*-ethylmaleimide and GSSG were added simultaneously, both at 0.5 mM, the degree of inhibition obtained was about

Fig. 2. Influence of pretreatment of glutathione reductase with NADPH on the inhibition by *N*-ethylmaleimide. Assay of enzyme activity was carried out at 20° in 0.1 M-phosphate buffer, pH 7.4 (1 mM with respect to EDTA, 0.8 mM with respect to GSSG and 0.1 mM with respect to NADPH). Degree of inhibition determined by measuring initial rate of reaction over the first 3 min. Below, (↓) denotes a period of waiting, duration of which is indicated on the abscissa, before reaction was initiated by the addition of GSSG or NADPH or both. The order of addition was: ×, Enzyme, *N*-ethylmaleimide (0.02 mM), NADPH, (↓), GSSG; ○, enzyme, NADPH (left 10 min.), *N*-ethylmaleimide (0.02 mM), (↓), GSSG; △, enzyme, *N*-ethylmaleimide (0.02 mM) (left 10 min.) NADPH, (↓), GSSG; □, enzyme, *N*-ethylmaleimide (0.02 mM), (↓), GSSG, NADPH; ●, enzyme, NADPH, *N*-ethylmaleimide (0.1 mM), (↓), GSSG; ▲, enzyme, NADPH, *N*-ethylmaleimide (0.5 mM), (↓), GSSG.

70–80 % and the reaction rate was linear after the first minute. When, however, to the same enzyme was first added *N*-ethylmaleimide and GSSG (the order of addition being immaterial), and the reaction was initiated by NADPH, no marked inhibition of the enzyme occurred for the first 2 min., but inhibition increased as the reaction proceeded and eventually reached the same level as in the first procedure (Fig. 3). In the first case *N*-ethylmaleimide and GSSG were added to an enzyme completely reduced; in the second case the presence of GSSG prevented complete reduction of the enzyme, hence the decreased rate of inhibition by *N*-ethylmaleimide.

Consistent with our interpretation of these findings is the fact that L-cystine, which is not reduced by the enzyme (Conn & Vennesland, 1951), did not inhibit the reduction of GSSG or protect the reduced enzyme against inactivation by *N*-ethylmaleimide. In these experiments the buffer solution was saturated with L-cystine to give an approximate concentration of 0.4 mM, GSSG being used in a concentration of 0.08 mM.

Inhibition by p-chloromercuribenzoate, arsenite and iodoacetate. We have so far made no assumption as to the nature of the group with which *N*-ethylmaleimide reacts. Maleic acid or its derivatives react rapidly with the sulphydryl groups of substances like GSH or L-cysteine (Gregory, 1955),

though less rapidly with protein sulphydryl groups (Roberts & Rouser, 1958). If a sulphydryl group were involved, we should expect to obtain a similar type of inhibition with other thiol reagents, e.g. the arsenicals and mercurials.

In studying the inhibition of the enzyme by *p*-chloromercuribenzoate the position is complicated by the fact that the resulting inhibition is readily reversed by GSH, which in this case is a product of the enzymic reaction. Thus the degree of inhibition produced by the mercurial has to be determined by assessing the rate of the reaction within the first minute, for, as GSH is formed, so the inhibition is rapidly reversed.

Inhibition of the enzyme under similar conditions to those described above for *N*-ethylmaleimide occurred with arsenite, iodoacetate and *p*-chloromercuribenzoate (Table 3). With the last-named inhibitor it was possible to treat the enzyme in the absence of NADPH with the mercurial at a concentration between 0.01 and 1 μ M for various periods and to observe no inhibition of activity on the addition of NADPH and GSSG. On the other hand, when NADPH and the mercurial were left in contact with the enzyme before the reaction was started by the addition of GSSG, marked inhibition (37–95 %) was observed. With higher concentrations (0.1–1 mM) of *p*-chloromercuribenzoate, apparent inhibition of the enzyme occurred even when there was no pretreatment of the enzyme

Fig. 3. Protective action of GSSG against inhibition of glutathione reductase by *N*-ethylmaleimide. Assay of enzyme activity was under the conditions described in Fig. 2. GSSG and *N*-ethylmaleimide were both used at a concentration of 0.5 mM. The order of addition was: ●, enzyme, NADPH, *N*-ethylmaleimide + GSSG; ○, enzyme, *N*-ethylmaleimide + GSSG, NADPH; △, enzyme, NADPH, GSSG.

Table 3. Inhibition of glutathione reductase by *p*-chloromercuribenzoate and arsenite, dependent on pretreatment of enzyme with reduced nicotinamide-adenine dinucleotide phosphate

The order of addition was: enzyme, inhibitor, NADPH (0.1 mM), GSSG (0.8 mM). Experimental details are given in the text.

Inhibitor	Concn. (μ M)	Time enzyme left in contact with inhibitor and NADPH before addition of GSSG (min.)	Percentage inhibition
<i>p</i> -Chloromercuribenzoate	0.01	0	0
	0.01	6	37
	0.05	0	0
	0.05	6	57
	0.1	0	0
	0.1	2	95
	0.1*	0	0
	(mM)		
Arsenite	1.0	0	0
	1.0	7	35
	1.0	12	45
	10	0.5	40
	10	2	78
	10	4	86
	10*	0	0

* Enzyme left in contact with inhibitor for 6 min.

with NADPH and inhibitor; this is the phenomenon observed by Asnis (1955) and by Langdon (1958). It may be due either to a very rapid reaction of the mercurial with the enzyme after the addition of NADPH, or possibly to a reaction between the mercurial and other protein sulphydryl groups not concerned in the enzymic activity, but which are necessary for maintaining the structural configuration of the enzyme molecule. With lower concentrations ($0.01 \mu\text{M}$) of *p*-chloromercuribenzoate the rate of reaction of the mercurial with the enzyme was slower, and thus inhibition occurred only after pretreatment with NADPH. This interpretation has been confirmed by an experiment in which the enzyme was treated with *p*-chloromercuribenzoate at a concentration of 0.1 mM for 10 min., dialysed for 2 days against frequent changes of phosphate-EDTA buffer and then tested. It retained complete activity, whereas a similar experiment in which the enzyme was first treated with NADPH and a much lower concentration ($0.05 \mu\text{M}$) of *p*-chloromercuribenzoate showed an almost complete (95%) loss of activity, an inhibition which was completely reversed during the progress of the reaction owing to the formation of GSH.

The results obtained with arsenite and iodoacetate can be interpreted on the same basis as those for *N*-ethylmaleimide and *p*-chloromercuribenzoate, i.e. that only the reduced enzyme reacts with the inhibitor. With arsenite complete reactivation of the treated enzyme occurred with 0.5 mM -2,3-dimercaptopropanol and partial reactivation with mM -GSH after the removal of excess of arsenite by dialysis.

Inhibition by allicin. Allicin is the sulphoxide of diallyl disulphide (Cavallito, Buck & Suter, 1944), and reacts rapidly with sulphydryl groups to give a disulphide which may be reduced by thiols. The presence of a sulphydryl group on the reduced enzyme was shown by the fact that it was inhibited (80% after 10 min.) by the sulphoxide (10 mM) and that the inhibition was reversed by GSH [20% reversal immediately on adding GSH (mM), rising to 80% reversal after 3 hr.]. The oxidized enzyme was not inhibited by allicin even when left in contact for a long period (16 hr.).

None of the reagents used above are absolutely specific for sulphydryl groups, and alkylating agents particularly are generally unreliable for the detection of these groups (Baron, 1951). Nevertheless, the evidence presented above is in general agreement with the hypothesis of the production of a sulphydryl group on the enzyme when this is reduced by NADPH. Thus the rate of reaction of *N*-ethylmaleimide with the reduced enzyme is relatively rapid; not, it is true, as fast as the reaction with GSH (Gregory, 1955), but certainly as rapid as the reaction of *N*-ethylmaleimide with

sulphydryl groups of other proteins (Roberts & Rouser, 1958), and much quicker than the reaction of *N*-ethylmaleimide with phosphoglucumutase, an enzyme which Milstein (1961) suggested contained no sulphydryl groups. Additional support for the view that a sulphydryl group is present is given by the observation that the reduced enzyme inhibited by *p*-chloromercuribenzoate is very rapidly reactivated by thiols such as GSH or L-cysteine. The sensitivity of the enzyme to Cu^{2+} and Zn^{2+} ions in the presence of NADPH is also explicable on this basis. In its oxidized form the enzyme is not inactivated by copper or zinc salts at a concentration of $0.1 \mu\text{M}$, but the reduced enzyme is rapidly and completely inactivated; this inactivation can be reversed by either GSH or L-cysteine (mM).

Reduction of the enzyme

Coenzyme required. Though previous work on the enzyme (Mapson & Goddard, 1951; Conn & Vennesland, 1951) suggested that only NADPH could function as coenzyme, we have now shown, using a concentrated enzyme preparation, that NADH can substitute for NADPH, though the rate of the reaction catalysed is about 100 times slower than with NADPH. This may explain why Mapson & Goddard (1951) failed to observe a reaction in experiments with much weaker enzyme preparations.

Other reducing agents. Sodium borohydride (tetrahydroborate), dithionite or sodium amalgam cannot replace NADPH. In particular, treatment of the enzyme with these reagents does not generate a sulphydryl group, which is a characteristic of the true reduced enzyme. In typical experiments the enzyme was pretreated with borohydride or dithionite at a concentration of mM for 30 min. at 20° in the presence of a thiol reagent and then dialysed against 0.1 M -phosphate buffer, pH 7.4 (1 mM with respect to EDTA), to remove excess of inhibitor. As both *N*-ethylmaleimide and *p*-chloromercuribenzoate react rapidly with dithionite, 0.01 mM -iodoacetic acid was used as the thiol reagent. In no circumstances after such treatments was any inhibition of the enzyme observed. The same result was obtained when the excess of dithionite was removed by aeration instead of by dialysis before the addition of the substrates. A similar experiment, in which the enzyme ($0.02 \mu\text{mole}$) was treated with dithionite ($0.2 \mu\text{mole}$) and then a large excess of *N*-ethylmaleimide ($0.5 \mu\text{mole}$) added to destroy the dithionite and act as inhibitor at the same time, showed no inhibition when GSSG and NADPH were added.

Stoichiometric relationship. Determinations of the minimum amount of NADPH to convert the oxidized enzyme into the reduced form and of the

minimum amount of GSSG to convert the reduced form into the oxidized state were carried out as follows. The oxidized enzyme was treated with an excess of NADPH and the resulting reaction products were removed by filtration through collodion sacs under nitrogen (see the Experimental section). The enzyme was again treated with excess of NADPH and the procedure repeated; the reduced enzyme was finally washed several times with dilute phosphate buffer solution. By this means the enzyme was converted into the reduced form and all substrates and products were removed. The reduced enzyme was then allowed contact with oxygen (aeration at room temperature) when there was a slow conversion into the oxidized form (shown by insensitivity to *N*-ethylmaleimide). This oxidized enzyme was used for the measurement of the stoichiometric relationship. Various amounts of NADPH were added, and then excess of *N*-ethylmaleimide and the residual activity of the enzyme measured, all operations being carried out under nitrogen to avoid reoxidation of any reduced enzyme. This remaining activity was directly proportional to the enzyme still in the oxidized state. The results (Fig. 4a) show that (by extrapolation) it was necessary to add 1 mole of NADPH/mole of oxidized enzyme to reduce it completely. Conversely (Fig. 4b), to

Fig. 4. (a) Amount of NADPH necessary to convert the oxidized form of glutathione reductase into the reduced form, as determined by the inhibition with *N*-ethylmaleimide. Oxidized enzyme ($14\mu\text{moles}$) and *N*-ethylmaleimide ($50\mu\text{moles}$) were used in 3 ml. of phosphate buffer. NADPH was added in various amounts (indicated on the abscissa) and the mixture left for 8 min. to allow maximum inhibition to develop before assay of enzyme activity. (b) Amount of GSSG necessary to convert the reduced enzyme into the oxidized form. The oxidized enzyme ($20\mu\text{moles}$) was reduced with excess of NADPH ($60\mu\text{moles}$), various amounts of GSSG were added and the sensitivity to *N*-ethylmaleimide was determined as in (a).

reconvert the reduced form into the oxidized form (shown by insensitivity towards *N*-ethylmaleimide) 1 mole of GSSG/mole of the reduced enzyme had to be added. In these latter experiments the reduced form was generated by the addition of a slight excess of NADPH, and GSSG gradually added. The excess of NADPH was first removed, as shown in Fig. 4b by the apparent lag phase, and then the reduced enzyme itself was oxidized.

Nature of the sulphur group on the oxidized enzyme

The possibility that the sulphhydryl group was produced by the reduction of a disulphide bond in the oxidized enzyme was rendered less probable by results of experiments in which the enzyme was pretreated with thioglycollate, a reagent which reduces many disulphide groups on proteins to sulphhydryl groups (Lindley, 1955). When the enzyme was treated with 0.1 M-sodium thioglycollate at pH 7.0 for 1 hr. at 20° , either in the presence or absence of 6 M-urea, and the excess of thioglycollate removed by dialysis against several changes of 0.1 M-phosphate buffer, pH 7.4 (1 mM with respect to EDTA), for 20 hr. under anaerobic conditions to prevent any reoxidation of sulphhydryl groups, the enzyme was not inhibited on the addition of 0.05 mM-*N*-ethylmaleimide. A comparable treatment with NADPH and dialysis under the same conditions gave an enzyme which was almost completely inhibited by *N*-ethylmaleimide, which indicates that the reduced enzyme was not being reoxidized during dialysis. In other experiments, in which the oxidized enzyme was treated with cyanide or sulphite (100 mM) at pH 7.6 for 20 min. in the presence of 0.5 mM-*N*-ethylmaleimide, the excess of these reagents being removed by dialysis before test, gave similar results. In these particular experiments a sulphhydryl group formed by cleavage of the disulphide bond might be expected to react with the thiol reagent and thus promote the complete cleavage of the disulphide bond. None of these experiments, however, give any support for the existence of a disulphide bond on or near the active centre of the oxidized enzyme.

The possibility that the presence of flavin situated close to such a disulphide bond in the enzyme might protect it was also examined. We repeated the above experiments on the apoenzyme (for preparation see below) and finally reconstituted the enzyme by the addition of FAD. Such experiments gave no evidence for the formation of a sulphhydryl group after reduction by cyanide, sulphite or thioglycollate.

The low affinity of arsenite, compared with either *p*-chloromercuribenzoate or *N*-ethylmaleimide, for the reduced enzyme also argues against the possibility that a disulphide group similar to

that of lipoic acid is involved, as does the fact that the inhibition by arsenite is reversed by GSH as well as by 2,3-dimercaptopropanol (Sanadi, Langley & White, 1959). It is also improbable that the failure of the oxidized enzyme to react with *N*-ethylmaleimide or other thiol reagents was due to a masking of a sulphydryl group by hydrogen bonding, for *N*-ethylmaleimide did not react even in the presence of concentrations of urea as high as 6–8 M, which this enzyme tolerates without irreversible inactivation. In these experiments the enzyme was pretreated with urea (6–8 M), either alone or with 0.05 mM-*N*-ethylmaleimide, for 20 min. at 20°. After removal of urea and excess of *N*-ethylmaleimide by dialysis against 0.01 M-phosphate buffer, pH 7.4 (1 mM with respect to EDTA), for 17 hr. at 1°, no inhibition of the enzyme was observed in either case. Similar experiments in which the enzyme was pretreated with 0.1 M-ammonium chloride in the presence or absence of *N*-ethylmaleimide gave similar results, a finding which suggests that formation of a thiazoline ring (Linderstrøm-Lang & Jacobsen, 1950) was not responsible for the masking of the sulphydryl group.

Inactivation of oxidized enzyme: treatment with benzoyl peroxide. Glutathione reductase, which in its oxidized state is immune to attack by thiol reagents, is inhibited by treatment with oxidizing agents such as benzoyl peroxide. This reagent possesses the advantage over many other oxidizing agents that owing to its insolubility the concentration of hydroperoxide in solution is kept at a constant low level. When the oxidized enzyme was treated with a saturated solution of the peroxide, left for 3 hr. at room temperature and dialysed to remove excess of the reagent, the enzyme was almost completely inhibited, but could be partially (25 %) reactivated by the addition of GSH (mM). In parallel experiments in which the peroxide was replaced by ammonium persulphate (mM), there was no inactivation of the enzyme. A possible explanation is that the benzoyl peroxide oxidizes the sulphur atom (cf. Small, Bailey & Cavallito, 1947) associated with the active group either to a sulfoxide or a disulphide which cannot be reduced by NADPH. Both sulfoxides and disulphides react with excess of thiol to give the original thiol, and so such a modified enzyme might be expected to be reactivated by the addition of GSH.

Inactivation of oxidized enzyme: treatment with either permanganate or mercuric oxide. Both alkaline permanganate and yellow mercuric oxide rapidly inactivated the oxidized form of the enzyme; either compound, when added to the oxidized enzyme in 0.1 mM concentration at room temperature, caused complete inactivation within a few minutes. Oxidation by alkaline permanganate was irreversible, but the enzyme inactivated by

mercuric oxide could be reactivated by treatment with hydrogen sulphide in dilute ammonia. In a typical experiment the enzyme (0.2 μ mole) treated with yellow mercuric oxide (0.03 μ mole) in 1 ml. of 0.01 M-tris buffer, pH 7.4, and allowed to stand for 10 min. before assay was completely inactivated. This inactivated enzyme was then treated for 3 min. with 1 ml. of a solution of ammonium sulphide, prepared by the addition of hydrogen sulphide to 4 M-ammonia in 0.01 M-tris buffer until the pH was decreased to 9.2, and the mixture centrifuged to remove mercuric sulphide. Almost complete restoration (75–90 %) of enzyme activity was achieved. Attempts to reactivate the enzyme inactivated by mercuric oxide with thiols, e.g. L-cysteine, were uniformly unsuccessful. These results are of interest because of their analogy with the reactions of thiourea under the same conditions (Werner, 1923), especially the ability of ammonium sulphide to regenerate the active enzyme, in contrast with the failure of thiols to do the same.

Nature of the intermediates formed in the enzymic reaction between oxidized glutathione and reduced nicotinamide-adenine dinucleotide phosphate

A study of the kinetics of the overall reaction indicates that it occurs in two stages (cf. equations 1 and 2), the link between the two stages being the reduced enzyme. This reduced enzyme carries a group that can react as a sulphydryl group, and when treated with a reagent like *N*-ethylmaleimide or *p*-chloromercuribenzoate the enzyme is inactivated. The oxidized enzyme, prepared by allowing the reduced enzyme (freed from all soluble substrates) contact with oxygen, required 1 mole of NADPH/mole to reduce it. Similarly the reduced enzyme required 1 mole of GSSG/mole to oxidize it. In each case the amount of reduced enzyme present was determined by the addition of *N*-ethylmaleimide and estimation of the enzyme that was not irreversibly inactivated. These facts agree with the outline formulation of the reaction as shown in equations (1) and (2), but do not indicate the nature of either the oxidized or reduced forms of the enzyme. In particular they do not indicate whether the oxidized enzyme contains some bound glutathione residues or NADP⁺, or whether the reduced enzyme contains bound NADPH. Some of these possibilities have been examined.

The first possibility, that bound glutathione residues might be present in the oxidized enzyme, has already been suggested by Langdon (1958) for yeast glutathione reductase. He pictured the reaction as shown in equations (3) and (4), in

which Enzyme·S·SG is the oxidized form and Enzyme·SH the reduced form. In this case, though the overall reaction agrees with the facts outlined above, it must be noticed that the product of the first stage would be 1 mole of GSH/mole, and of the second also 1 mole of GSH/mole.

The second possibility is that no substrates are bound, the reaction sequence being as shown in equations (5) and (6). The product of the first stage

would contain no GSH but the product of the second stage would contain 2 moles of GSH/mole. Because of this difference between the two types of reaction sequence, we have used the production of GSH from both the oxidized and reduced forms of the enzyme as an indication of their structure. To isolate the small quantities of GSH which would be formed, we have treated either the oxidized or the reduced forms of the enzyme in 0.01 M-phosphate buffer, pH 7.4 (1 mM with respect to EDTA), with either NADPH or GSSG and then separated the enzyme by filtering the solution in an atmosphere of nitrogen through a collodion membrane. The filtrate contained the GSH and other low-molecular-weight material.

Pretreatment of the enzyme with oxidized glutathione and its effect on the formation of reduced glutathione on subsequent addition of reduced nicotinamide-adenine dinucleotide phosphate. The amount of GSH liberated, when the oxidized enzyme was treated with NADPH in excess, depended on the amount of GSSG added initially to the reduced enzyme to convert it into the oxidized form. When GSSG was added at a level of up to 16 moles of GSSG/mole of reduced enzyme and the excess of GSSG and GSH formed removed by filtration and washing, there was no production of GSH on subsequent addition of NADPH to the treated enzyme. However, as the proportion of GSSG to reduced enzyme was increased, the amount of GSH liberated on subsequent treatment with NADPH rose to 2 moles/mole of enzyme. Such results show that when the reduced enzyme is treated with a small excess of GSSG the only reaction is with the active catalytic site of the enzyme. It seems possible that this reaction may involve the intermediary formation of a mixed disulphide between Enzyme·SH and GSSG, e.g. Enzyme·S·SG, and proceeds to completion with the transference of two electrons from the reduced active centre to give complete reduction of 1 mole of GSSG. Under these circumstances the enzyme

only exists momentarily in the Enzyme·S·SG form, the stable oxidized form of the enzyme containing no mixed disulphide group. When, however, the reduced enzyme is treated with a larger excess of GSSG, in addition to the reaction with the active centre, GSSG becomes bound to the enzyme on a secondary site. The GSSG so bound is able to react with the active centre when this is reduced on subsequent treatment with NADPH, thereby resulting in the liberation of GSH.

It is difficult from these experiments to be certain how many moles of GSSG/mole must be added in excess of that required to oxidize the reduced enzyme before any is bound on a secondary site. When the reduced enzyme was treated with excess of GSSG, it was necessary to wash the enzyme protein three times to ensure the complete removal of free GSSG. Under such circumstances GSSG might well dissociate from such a site, as the concentration of GSSG in solution is decreased by washing. Our results do no more than indicate that GSSG may be bound on the oxidized enzyme and that the GSSG so bound can react with the active centre when this is reduced by NADPH.

NADH can substitute for NADPH in these reactions and this is in agreement with the results reported above.

The oxidized enzyme, prepared from the reduced enzyme (freed from soluble substrates) by allowing contact with oxygen, did not produce any GSH when it was reduced with NADPH, so that in this case, as with the reduced enzyme oxidized with small amounts of GSSG, no glutathione residues are bound to the enzyme.

Pretreatment of the enzyme with reduced nicotinamide-adenine dinucleotide phosphate and its effect on the formation of reduced glutathione on subsequent addition of oxidized glutathione. The oxidized enzyme was treated with an excess of NADPH (the amount varied in different experiments from 25 to 100 moles/mole of enzyme) and the reduced enzyme was isolated by filtration. Oxygen was excluded by carrying out all operations in an atmosphere of nitrogen. This reduced enzyme was then treated with an excess of GSSG (13–160 moles/mole of enzyme), and both the GSH and NADP⁺ formed were measured. In all cases, after three washings of the reduced enzyme, 18 moles of GSH/mole and 8 moles of NADP⁺/mole were formed when GSSG was added to the enzyme. After six washings the amount of GSH and NADP⁺ liberated had fallen to such a level that it was impossible to determine accurately the proportion between the two, a ratio of between 1 and 2 moles of NADP⁺ to 4 moles of GSH being estimated. With ten washings the enzyme had reverted to the oxidized form before any GSSG was added, probably as a result of the difficulty of excluding all

traces of oxygen during the manipulations. Three washings were normally sufficient to decrease the concentrations of substances freely soluble in the buffer to a negligible level. These results therefore suggest that, though the enzyme is able to bind by physical adsorption or complex-formation additional moles of NADPH/mole over that required to reduce the catalytic centre, such NADPH is removed by washing. The binding of 8 moles of NADPH/mole of enzyme represented the maximum amount which could be held in this way, and thus on the addition of GSSG 16 moles of GSH/mole could be released plus a further 2 moles/mole by oxidation of the active centre.

Binding of oxidized [^{35}S]glutathione to the oxidized enzyme

Experiments were carried out with [^{35}S]GSSG to obtain more direct evidence that GSSG can combine with the oxidized form of the enzyme. In all cases the enzyme (0.3 $\mu\text{m-mole}$) in 1 ml. of 0.1 M-phosphate buffer, pH 7.4; was treated with [^{35}S]GSSG (5 $\mu\text{m-moles}$; 0.053 μC) and allowed to stand for 10 min. at room temperature. The treated enzyme was then isolated in various ways and the radioactivity of the [^{35}S]GSSG attached to the protein measured.

Enzyme precipitated by acetone. Acetone (at -20°) was added with stirring to the chilled solution to give a final concentration of 50% (v/v), and the precipitated enzyme centrifuged down and washed in the centrifuge with chilled aq. 75% (v/v) acetone three times. It was dissolved in water (2 ml.), frozen at -20° and then freeze-dried. The dry powder was dissolved in water (0.2 ml.) and a sample (0.05 ml.) taken for the measurement of radioactivity. The whole protein had 49 counts/min., which with a counting efficiency of 1.56% was equivalent to the binding of 0.56 mole of GSSG/mole of enzyme. The remaining solution (0.15 ml.) was treated with 0.1 mM-NADPH, left for 10 min. at room temperature, and then heated at 100° for 2 min. to denature the enzyme. After the protein precipitate had been removed by centrifuging, the radioactivity of the supernatant was equivalent to 38 counts/min. for the whole enzyme (0.44 mole of GSSG/mole of reduced enzyme). This meant that 78% of the original GSSG bound to the protein had been released by NADPH.

Enzyme separated by electrophoresis. Electrophoresis of the solution on Whatman no. 3 paper, with 0.1 M-phosphate buffer, pH 7.4, as electrolyte, easily separated free GSSG from the protein. A moist-chamber type of apparatus was used (Laurell, 1957) with the paper stretched horizontally. At a potential gradient of 3 v/cm. and a current of 3 ma a clear-cut separation was ob-

tained in 17 hr. at 1° . The position of the enzyme was found by staining a test strip with bromophenol blue. The remainder of the enzyme was eluted from the paper by washing with water, and the solution was freeze-dried. Measurement of the radioactivity indicated that the isolated protein had 57 counts/min., equivalent to 0.65 mole of GSSG/mole of enzyme.

Enzyme separated by ultracentrifuging. Three separate solutions were prepared: (1) [^{35}S]GSSG (5 $\mu\text{m-moles}$; 0.053 μC) in 1 ml. of 0.1 M-phosphate buffer, pH 7.4; (2) as (1) but with enzyme (0.33 $\mu\text{m-mole}$); (3) as (2) but with NADPH (50 $\mu\text{m-moles}$). The three solutions were centrifuged at 170 000g for 5 hr. in a Spinco centrifuge and then samples (0.05 ml.) removed very carefully from the top, middle and lower layers of each with a micrometer syringe. The results, in the terms of the counts/min. of the samples, are shown in Fig. 5. Only in solution (2) was there a definite gradation of activity from top to bottom; this corresponds to the fact that there was about twice as much enzyme activity in the bottom layer as there was at the top, in spite of some loss of total enzyme activity during the experiment.

Enzyme precipitated by ammonium sulphate. Solid ammonium sulphate (500 mg.) was added and, after standing for 15 min. at 0° , the precipitated enzyme was centrifuged down and washed three times on the centrifuge with cold saturated ammonium sulphate (1 ml. each time). It was then dissolved in 0.2 ml. of 0.05 N-sodium hydroxide and a sample (0.05 ml.) taken for measurement of

Fig. 5. Effect of ultracentrifuging on the distribution of radioactivity in a solution of labelled GSSG and the oxidized form of glutathione reductase. Solution (1): GSSG. Solution (2): GSSG + enzyme. Solution (3): GSSG + enzyme + NADPH. The amounts used (in 1 ml. total volume) were: GSSG, 5 $\mu\text{m-moles}$; enzyme, 0.33 $\mu\text{m-mole}$; NADPH, 50 $\mu\text{m-moles}$. The solutions were centrifuged in a Spinco centrifuge at 170 000g for 5 hr. The numbers in the Figure refer to counts/min. obtained on 0.05 ml. samples taken from the appropriate positions.

radioactivity. The treated enzyme protein prepared in this way had a negligible radioactivity (less than 1 count/min.). Thus from comparison with the results described above with other methods for isolating the enzyme, ammonium sulphate appears to split the link binding GSSG to the oxidized enzyme.

Prosthetic group

The prosthetic group of the enzyme is FAD. This was demonstrated by (a) the separation of the apoenzyme from the flavin by acid treatment in the presence of ammonium sulphate and its reactivation by the addition of FAD, and (b) the reactivation of the apoenzyme of D-amino acid oxidase by the flavin split off from the enzyme. The preparation of the apoenzyme and its reactivation by FAD, and the ineffectiveness of both FMN and riboflavin, have been reported by Mapson (1959).

Spectrum of the enzyme: semiquinone formation. The most active preparations of the plant enzyme are yellow, show yellowish fluorescence in ultra-violet light, and on reduction with NADPH turn red, a phenomenon which has been observed with other flavoproteins and has been ascribed to the formation of a semiquinone (Beinert, 1956). The spectrum of the oxidized form of the enzyme is typical of many flavoproteins, showing maxima at 375, 456 and 487 $m\mu$ (Fig. 6), and the changes in the spectrum on the addition of NADPH are similar to those reported by Massey *et al.* (1960) for lipoate dehydrogenase and believed by them to be due to semiquinone formation. NADH or dithionite also reduce the flavin, to give a similar spectrum to that obtained with NADPH. The former observation is not surprising since we have shown that NADH can act as substrate in the reaction, but the observation with dithionite was unexpected since with many flavoproteins the flavin is completely reduced (Dolin, 1957; Massey *et al.* 1960). A fully reduced flavin spectrum was not obtained even when dithionite was present in marked excess and the treatment prolonged for periods of up to 1 hr. under anaerobic conditions.

Dolin observed this state of affairs with his flavoprotein peroxidase only when NADH or NAD^+ was present. The enzyme used in our experiments had been pretreated with an excess of GSSG and subsequently dialysed (2 ml. of enzyme against two changes of 500 ml. of buffer for 8 hr.) to oxidize any NADPH and remove $NADP^+$. Despite this extensive dialysis it is possible that $NADP^+$ may be still attached to the enzyme, the explanation for the spectrum obtained with dithionite being that the $NADP^+$ -flavoprotein complex was reduced by dithionite to give a spectrum identical with that obtained with NADPH. However, if this is the correct interpretation it is difficult to under-

stand why dithionite, in reducing the flavin, does not generate a sulphhydryl group as is the case with either NADPH or NADH.

Spectrum of the enzyme: effect of the addition of mercurials. Massey *et al.* (1960) observed changes in the spectrum of the reduced form of lipoate dehydrogenase after the addition of *p*-chloromercuribenzenesulphonate. The changes reported by them are generally similar to those we have observed with the reduced form of glutathione reductase on the addition of *p*-chloromercuribenzoate (Fig. 7). There was a fairly rapid reaction after the addition of the mercurial, which caused a general decrease in absorption over the range

Fig. 6. Absorption spectra of the oxidized and reduced forms of glutathione reductase. Enzyme (23 μ m-moles) was dissolved in 1 ml. of 0.1M-phosphate buffer, pH 7.4. Broken line: absorption spectrum of oxidized form. Continuous line: absorption spectrum of reduced form, obtained after the addition of NADPH (0.1 μ mole).

Fig. 7. Effect of *p*-chloromercuribenzoate on the absorption spectra of the oxidized and reduced forms of glutathione reductase. Enzyme (30 μ m-moles) was dissolved in 1 ml. of phosphate buffer, pH 7.4. Broken line: absorption spectrum of oxidized form. Continuous lines: absorption spectra of reduced form, obtained after the addition of NADPH (0.2 μ mole): ○, without further treatment; ●, 5 min. after the addition of *p*-chloromercuribenzoate (0.1 μ mole); ×, after oxidation by air.

400–600 $m\mu$. The absorption band at about 530 $m\mu$ practically disappeared, a phenomenon also observed by Massey *et al.* (1960) with lipoate dehydrogenase and attributed by them to the presence in the enzyme of a flavin semiquinone stabilized by a sulphhydryl group. When the sulphhydryl group reacts with the mercurial, the semiquinone is no longer stabilized and practically disappears.

We also examined the change produced in the spectrum of the oxidized form of glutathione reductase when the reduced enzyme treated with *p*-chloromercuribenzoate was allowed to reoxidize by contact with oxygen. Under these conditions an oxidized spectrum (Fig. 7) was obtained in which the three flavin maxima were shifted 7–14 $m\mu$ to shorter wavelengths.

Massey *et al.* (1960) reported that with lipoate dehydrogenase no reduction of the flavin occurred when the enzyme was pretreated with the mercurial. With glutathione reductase the flavin was reduced by NADPH as easily in the presence as in the absence of the mercurial. However, in the absence of *p*-chloromercuribenzoate, reduction of the enzyme by NADPH resulted in an increased absorption over the range 500–600 $m\mu$, which was not observed when the enzyme was pretreated with *p*-chloromercuribenzoate before the addition of NADPH. No change in the absorption of the oxidized form of the enzyme was observed on the addition of the mercurial, which is in agreement with the findings reported above that the mercurial does not react with this form of the enzyme.

Experiments with the apoenzyme. We decided to determine if the sulphhydryl group, that we have shown to be necessary for the enzymic reaction, was also present in the apoenzyme, or if such a group was formed on treatment with NADPH. The apoenzyme was precipitated from an acid solution (pH 2.5) of the reduced form of the enzyme by treatment with ammonium sulphate (50 % saturation) and separated by centrifuging. It was dissolved in 0.1 M-phosphate buffer, pH 7.4, to which either of the thiol reagents *N*-ethylmaleimide (0.5 mM) or arsenite (5 mM) was added, and the solutions were left to stand for 5–10 min. at room temperature. FAD (15 μ M) was then added, followed by GSSG and NADPH, and the enzymic activity determined. There was no difference in the initial activity of the apoenzyme protein so treated and a control apoenzyme when both were converted into the holoenzyme by the addition of FAD. In further experiments the apoenzyme was first treated with NADPH and left for 5 min. with the thiol reagents before GSSG and FAD were added; again there was no evidence of any immediate inhibition due to the thiol reagents. These results show that the sulphhydryl group is

neither found on the apoenzyme prepared from the reduced enzyme nor formed on the apoenzyme in circumstances in which it is formed on the flavoprotein. These results show that the presence of flavin is necessary for the formation of the sulphhydryl group.

Further experiments were made on an apoenzyme in which the sulphhydryl group was combined with a mercurial. The enzyme (0.02 mM) was reduced with NADPH in the presence of *p*-chloromercuribenzoate (0.1 mM) and the flavin subsequently split off by acidification in the presence of ammonium sulphate. The apoenzyme treated with *p*-chloromercuribenzoate could be reactivated by the addition of FAD and a thiol such as L-cysteine or GSH. The following experiment shows that flavin will recombine with this apoenzyme treated with *p*-chloromercuribenzoate. FAD (0.02 mM) was added to the apoenzyme treated with *p*-chloromercuribenzoate, the excess being removed by extensive dialysis (30 hr. against several changes of 0.01 M-phosphate buffer, 1 mM with respect to EDTA and 0.1 mM with respect to *p*-chloromercuribenzoate). The enzyme so treated was inactive but could be immediately reactivated by the addition of either L-cysteine or GSH. It seems clear that the integrity of the sulphhydryl group on the active centre is neither essential nor concerned with the binding of FAD to the apoenzyme. Moreover, the fact that a sulphhydryl group cannot be shown to exist on an apoenzyme prepared from the reduced enzyme indicates that the sulphhydryl group becomes oxidized, or disappears in some way, under the conditions which promote dissociation of the flavin. Whether this change is due to the acid conditions or to the dissociation of the flavin remains to be determined.

Inhibition by salts

The activity of the enzyme can be markedly affected by the presence of certain salts, particularly sodium iodide, in the solution, the magnitude of the effect depending on the concentration of the salt and especially on whether the enzyme is mainly in the oxidized or the reduced state. Since the effects observed with the oxidized enzyme are susceptible to a much simpler explanation and appear to be of a different type from those observed with the reduced enzyme, they will be described first.

Oxidized enzyme. Various salts were added to a standard reaction mixture containing enzyme (4 μ M) and GSSG (0.8 mM), and after 5 min. NADPH (0.1 mM) was added and the reaction velocity measured. Under the conditions of the experiment, the inhibition did not increase as the reaction proceeded, and, moreover, the salt effect was completely reversible, for dialysis of the

enzyme digest against buffer solution restored the full activity of the enzyme. The decrease in rate due to the addition of the salt was calculated as a percentage of the full activity when no salt was present, and in Table 4 is referred to as the percentage inhibition. The halide salts are especially interesting in that the percentage inhibition increases with the size of the anion ($\text{Cl}^- < \text{Br}^- < \text{I}^-$).

In later experiments a detailed study was made of the kinetics of the inhibited enzyme reaction with sodium iodide as the salt; sodium iodide was chosen because it showed the strongest inhibitory effect. The relationship between enzyme reaction velocity, v , substrate (GSSG) concentration, S , and inhibitor concentration, i , was analysed by plotting $1/v$ against $1/S$ (Lineweaver & Burk, 1934), $1/v$ against i , and v against v/S (Dixon, 1953). The graphs obtained all indicate that the relationship between sodium iodide and GSSG is of a competitive type. The plot of $1/v$ against i is shown in Fig. 8, from which the dissociation constant K_i for sodium iodide was calculated to be 23 mM, i.e. an affinity of 0.43, which is 1000 times less than that of GSSG. These results therefore suggest that sodium iodide inhibits the enzyme in the oxidized state by competing for a site which is concerned with the binding of GSSG. The fact that the inhibition by salts (even sodium iodide, 0.5 M) is completely reversible indicates that there has been no loss of flavin.

Reduced enzyme. The various salts were added to a standard reaction mixture containing enzyme (4 μM) and NADPH (0.1 mM), and then after various times GSSG (0.8 mM) was added and the reaction velocity measured. The results show that the inhibition produced was the same in all cases as with the oxidized enzyme except for iodides. The presence of even small amounts of iodide had a unique effect on the reduced enzyme; the inhibition was much greater than when the iodide was added to the oxidized enzyme, and increased if the iodide was left with the reduced enzyme for up to 8 min. before initiating the reaction by adding GSSG. Thus, in a typical experiment when sodium iodide (final concn. 0.05 M) was added to the reduced enzyme, the reaction was inhibited 88 % as compared with 42 % when the sodium iodide was added to the oxidized enzyme. This increase in inhibition (46 %) appeared to be superimposed on and of a different nature from that observed with the oxidized enzyme. The addition of GSH (mM), L-cysteine (mM), diethyldithiocarbamate (mM), potassium cyanide (10 mM) or sodium sulphite (10 mM) to the enzyme digest before the sodium iodide prevented the increase in the inhibition, though these compounds had no effect on the inhibition of the oxidized enzyme by the salt. The increase in the inhibition was also completely

reversed by the subsequent addition to the digest of GSH (mM), L-cysteine (mM) or diethyldithiocarbamate (mM) and partially reversed (45–60 %) by potassium cyanide (10 mM) and sodium sulphite (10 mM). The addition of *N*-ethylmaleimide (0.5 mM) and sodium iodide (0.05 M) simultaneously to the reduced enzyme, followed by dialysis to remove the reagents, gave an enzyme which was inactive but which could be largely reactivated (60 %) by the addition of GSH (mM). The failure to

Table 4. *Inhibition of glutathione reductase by salts*

Experimental details are given in the text.

Salt	Concn. (M)	Percentage inhibition
Glycine	0.5	0
Ammonium chloride	0.5	50
Sodium acetate	0.5	0
Sodium phosphate	0.5	30
Sodium sulphate	0.5	86
Sodium oxalate	0.2	58
Sodium nitrate	0.25	79
	0.5	100
Sodium chloride	0.25	0
	0.5	58
Sodium bromide	0.25	41
	0.5	71
Sodium iodide	0.25	97
	0.5	100

Fig. 8. Competitive inhibition of glutathione reductase between sodium iodide and GSSG. NaI was left in contact with the oxidized form of the enzyme and GSSG in a volume of 3 ml. of 0.1 M-phosphate buffer, pH 7.4 (1 mM with respect to EDTA), for 5 min. before the reaction was initiated by the addition of 0.1 mM-NADPH. Concn. of GSSG: $\times$, 50 μM ; $\circ$, 37 μM ; Δ , 13 μM . K_i was 23 mM.

obtain complete reactivation was possibly due to the fact that the iodide reaction with the sulphhydryl group was not fast enough to prevent some reacting with the *N*-ethylmaleimide. The evidence suggests strongly that a sulphhydryl group is destroyed with the concomitant formation of a disulphide link and that in some way the I^- ion is acting as a catalyst. If this is so the most likely oxidant in the system is atmospheric oxygen. It can be calculated that in a solution in contact with the air the oxygen present can reach a concentration of 50 mM, which is very much greater than the concentration of enzyme in the same solution (4 μ M), and means that a catalytic oxidation would be difficult to prevent except by the most rigorous exclusion of oxygen. Catalysis by heavy-metal ions present as impurities in the sodium iodide seems unlikely for the increase in inhibition due to sodium iodide was not prevented by the addition of chelating agents such as azide (10 mM), 1-nitroso-2-naphthol (0.1 mM), ammonium *N*-nitrosophenylhydroxylamine (mM), EDTA (mM), 8-hydroxyquinoline (mM), *o*-phenanthroline (mM) or salicylaldehyde (mM). The increase in inhibition was not due to the presence of iodate or iodine in the sodium iodide, for iodate (0.1 mM) had no inhibiting effect and the inclusion of ascorbic acid (mM) in the solution with the sodium iodide did not prevent the increase. If, however, only a minute trace of iodine is needed to act as a catalyst in the oxidation of the enzyme sulphhydryl group, the effectiveness of adding ascorbic acid is doubtful since dehydroascorbic acid will be in equilibrium with a trace of iodine in the solution. An approximate calculation from the redox potentials of I^- -iodine and ascorbic acid-dehydroascorbic acid at pH 7 (Dixon, 1949) indicates that the addition of the ascorbic acid would only lessen the rate of increase in inhibition by a factor of 5 and it is possible that the dehydroascorbic acid itself will readily oxidize the enzyme sulphhydryl group.

A summary of the experimental observations is given in Scheme 1. We have assumed that the enzyme contains a single sulphur atom which gives a sulphhydryl group when the enzyme is reduced with NADPH. The importance of Scheme 1 is in connexion with the suggestion already tentatively put forward that the sulphur atom is part of a

substituted thiourea group. Iodine very readily oxidizes thiourea in dilute solution (less than 0.04 %) at neutrality to formamidine disulphide $H_2\ddot{N}:C(NH_2)\cdot S\cdot S\cdot C(NH_2):\ddot{N}H_2$, whereas other oxidizing agents do not react in this way; iodine is unique in this respect (Werner, 1912). Thus we might expect the small amounts of iodine produced by the oxidation of sodium iodide in solution in the presence of oxygen to oxidize the sulphhydryl group of the enzyme to a disulphide analogous to formamidine disulphide and the enzyme to be reactivated by the regeneration of the sulphhydryl group on the addition of GSH or cysteine.

The addition of sodium iodide to the reduced enzyme can also promote the dissociation of the flavin. A series of experiments investigating this possibility is described in Table 5. The results show that weak solutions (0.05 M) inactivate the enzyme but that the activity is restored by the addition of GSH alone, FAD not being necessary; no dissociation of flavin therefore occurs. With higher concentrations (0.5 M) both FAD and GSH are necessary. This ability of the I^- ion to promote the dissociation of the flavin is increased in the presence of ammonium sulphate. Sodium iodide at even the lowest concentration (0.05 M) in the presence of ammonium sulphate (3 M) caused a small dissociation of flavin and this was increased when the concentration of sodium iodide was raised to 1 M. Further, in all experiments in which sodium iodide was used in concentrations of 0.5 M and above, dissociation of the flavin was always accompanied by a considerable irreversible loss of enzyme activity, which was not prevented by the presence of GSH. Whether this is linked with the oxidation of the sulphhydryl group to a disulphide is not clear but seems unlikely since GSH did not prevent either the dissociation or the loss.

DISCUSSION

The kinetic results are consistent with the view that the enzyme exists in two different oxidative states, with one substrate reacting with the oxidized and the other with the reduced form of the enzyme. We have also shown that to convert 1 mole of the oxidized form of the enzyme into the reduced state, 1 mole of NADPH is necessary; and

Scheme 1

Table 5. *Inhibiting effects of sodium iodide on the reduced form of glutathione reductase*

The final concentrations of added FAD and GSH were 0.4 and 1 mM respectively. Experimental details are given in the text.

Expt. no.	Treatment	Activity after treatment (% of original)	First addition	Activity after addition (% of original)	Second addition	Activity after addition (% of original)
1	NaI (0.5M) and dialysed	0	FAD	8	GSH	46
		0	GSH	28	FAD	40
2	NaI (0.5M) and dialysed	0	GSH	0	FAD	34
		0	FAD	10	GSH	46
3	NaI (0.5M) + GSH (mM) and dialysed	23	FAD	31	GSH	38
4	NaI (0.05M) and dialysed	8	FAD	8	GSH	75
		8	GSH	74	—	—
5	Enzyme precipitated with (NH ₄) ₂ SO ₄ (3M)	90	FAD	90	—	—
6	Enzyme + NaI (0.05M) and precipitated with (NH ₄) ₂ SO ₄ (3M)	49	FAD	65	GSH	97
		49	GSH	79	FAD	97
7	Enzyme + NaI (m) and precipitated with (NH ₄) ₂ SO ₄ (3M)	26	GSH	26	FAD	51
	Enzyme + NaI (m) + GSH (mM) precipitated with (NH ₄) ₂ SO ₄ (3M)	46	FAD	54	—	—

conversely that 1 mole of GSSG is necessary to convert the reduced into the oxidized form.

A sulphydryl group is generated on the enzyme when this is reduced by NADPH or NADH. The sensitivity of the reduced enzyme to thiol enzyme reagents, and to metal ions such as Cu²⁺ and Zn²⁺, and the conditions under which the enzyme may be reactivated, are all explicable on this basis. The formation of the sulphydryl group appears to be dependent on the presence of flavin, for in no circumstances have we detected a sulphydryl group on the oxidized enzyme, on the apoenzyme formed from the reduced enzyme, or on the apoenzyme after treatment with NADPH. In one experiment the oxidized enzyme was left with *N*-ethylmaleimide for a prolonged period (17 hr. at room temperature followed by dialysis) without the slightest indication of any inactivation. The oxidized form of the sulphur group does not seem to be that of a disulphide, the failure to regenerate a sulphydryl group after treatment with reagents such as thioglycollate, cyanide, sulphite or cysteine all pointing to this conclusion. Rather our evidence suggests that the sulphur atom may be present in a substituted thiourea grouping. Thus the oxidized enzyme is inactivated by benzoyl peroxide, permanganate and mercuric oxide, and the enzyme inactivated by mercuric oxide is regenerated by subsequent treatment with ammonium sulphide. This is characteristic of thiourea, which with the first two reagents is converted into urea and with mercuric oxide into cyanamide. Treatment of cyanamide with ammonium sulphide gives thiourea back again but the oxidation to urea is not reversible. The acyl derivatives of thiourea behave like thiourea towards mercuric oxide, the carbodi-imide derivatives being reconverted by ammonium

sulphide into the corresponding thiourea compound.

The oxidized form of the enzyme does not, as suggested by Langdon (1958), contain a GSH moiety present as Enzyme·S·SG, for no GSH is liberated on treatment of the oxidized enzyme with NADPH, and the same oxidized enzyme may be obtained by oxidation of the reduced form with oxygen without the production of GSH. Our evidence does not, however, exclude the possibility that a mixed disulphide may be formed as a labile intermediary in the reaction sequence.

We have confirmed the claim by Mapson (1959) that the prosthetic group of the enzyme is FAD, and have further shown that the sulphydryl group generated on the active centre of the reduced enzyme is not essential for or concerned with the binding of flavin to the apoenzyme. The observation that the yellow oxidized enzyme changes in colour to pink when NADPH is added and the effect of *p*-chloromercuribenzoate in modifying the spectrum of the reduced enzyme (especially that portion which has been ascribed to the formation of flavin semiquinones) but not affecting the spectrum of the oxidized enzyme, is consistent with the view that the sulphydryl group is essential for the establishment of a flavin semiquinone. On the other hand a spectrum apparently identical with that produced by the addition of NADPH, and yet not producing a sulphydryl group, argues against this possibility unless the assumption is also made that dithionite reacts with this sulphydryl group or prevents its reaction with thiol reagents such as iodoacetate or *N*-ethylmaleimide.

We have attempted to explain these results in terms of a working hypothesis based on the

Scheme 2

assumption that the thiourea moiety and the flavin are in close proximity. The oxidized and reduced enzyme (at pH 7) are then pictured as shown in Scheme 2. The acylated thiourea moiety in the oxidized enzyme would most probably exist in the non-zwitterionic form for analogous compounds such as parabanic acid, pK_a 6.1, hydantoin, pK_a 9.1, and thiohydantoin actually behave as weak acids. It seems clear that an acylated amide group would accept a proton with much greater difficulty than the corresponding unsubstituted group and at neutrality would probably be uncharged. The thiourea moiety in Scheme 2 has therefore been pictured in the uncharged form.

Parabanic acid

Hydantoin

Thiohydantoin

The particular property of the flavin which attracted our attention was the fact that the pK_a of the second dissociation constant of the isoalloxazine ring (position 4) changed from 10.4 in oxidized FAD to 6.7 in the reduced form (Lowe & Clark, 1956). By analogy with the corresponding values for riboflavin (Michaelis, Schubert & Smythe, 1936; Michaelis & Schwartsenbach, 1938), the semiquinone form would have pK_a 6.5. We have therefore suggested that the oxygen atom at position 4 when the flavin is reduced loses a proton (at pH 7.4) and the proximity of the negatively charged oxygen atom to the thiourea moiety induces it to assume the zwitterionic form in which the sulphur atom is negatively charged. The proximity of this sulphur atom to the NH group at position 10 on the reduced flavin causes the

hydrogen atom attached to the nitrogen atom to be shared between the two. It is tempting to assume that the reduced flavin then assumes many of the properties attributed to the semiquinone form. This picture is admittedly speculative but it does offer a plausible explanation for our experimental results. If we consider the oxidized form of the enzyme first, such a structure would explain the lack of reactivity towards *p*-chloromercuribenzoate, *N*-ethylmaleimide and similar thiol reagents, and for the fact that treatment with excess of thiols does not generate a sulphydryl group at the active centre. Since the thiourea moiety is not directly linked with the flavin, it would explain why blocking the sulphur atom in the apoenzyme by combination with *p*-chloromercuribenzoate (reduced enzyme treated with *p*-chloromercuribenzoate and flavin dissociated in acid solution), has only a small effect on the binding of FAD to the apoenzyme. It would also explain the fact that oxidation of the sulphur atom in the intact enzyme with benzoyl peroxide causes inactivation but not dissociation of the flavin. This inactivation can be reversed by the addition of thiols, and the most likely explanation is that either a sulfoxide or disulphide is formed at the active centre which is then reduced back to the original sulphur atom in the presence of thiols. The structure postulated for the reduced enzyme offers a ready explanation for its reactivity towards heavy-metal ions, *p*-chloromercuribenzoate, *N*-ethylmaleimide and iodine, and, if we accept that the stabilization of the so-called semiquinone form of the flavin is bound up with the integrity of the sulphydryl group and the charge structure of the thiourea moiety, then any chemical change in this group will destroy the semiquinone form. Treatment of the reduced enzyme with *p*-chloromercuribenzoate markedly changes the spectrum at wavelengths greater than 500 m μ so as to destroy the broad absorption band

present, which by analogy with the work of Massey *et al.* (1960) is due to the presence of a flavin semiquinone.

Perhaps the most striking characteristic of glutathione reduction is its absolute specificity towards GSSG. Our studies have revealed no precise reason for this. We have in this paper obtained some evidence that GSSG may be bound to the oxidized enzyme and can then react with the sulphhydryl group generated on the addition of NADPH. We have not, however, been able to demonstrate that GSSG must first be bound to a site on the oxidized enzyme before it can react with the sulphhydryl group of the reduced enzyme. If this were the case then the high specificity of the enzyme might be explicable on the theory that GSSG must first be orientated in an alignment on the enzyme surface in order to be in a position to participate in this transfer of electrons from the flavin. The specificity, on this view, resides in the positioning of the disulphide group relative to the active centre. Other disulphides are not reduced because of their inability to be orientated in this manner.

SUMMARY

1. The preparation and some properties of glutathione reductase, prepared from germinated peas, are described. Confirmation that the enzyme is a flavoprotein containing FAD as the prosthetic group was obtained.

2. The kinetic results are consistent with the view that the enzyme exists in two different oxidative states, with one substrate (NADPH) reacting with the oxidized form, and the other (GSSG) with the reduced form of the enzyme.

3. The addition to the apoenzyme of both FAD and either NADPH or NADH is required for the formation of a sulphhydryl group on the active centre. In the reduced state the enzyme is readily inactivated by compounds which react with sulphhydryl groups. The integrity of this sulphhydryl group is not essential for, or concerned with, the binding of FAD to the apoenzyme.

4. The oxidized form of the sulphhydryl group is not that of a disulphide, and the fact that GSH is not liberated on treatment with NADPH shows that a disulphide group of the type Enzyme-S-SG is not present, although such a group may be formed as an intermediate during the transfer of the two electrons from the FAD to GSSG.

5. The inhibition of the oxidized enzyme by oxidizing agents such as mercuric oxide, and the reactivation of the enzyme so treated with ammonium sulphide but not with thiols, suggest that a lone sulphur atom similar to that existing in thiourea is present.

6. The observation that the yellow oxidized

enzyme changes in colour to pink when NADPH is added, and the effect of *p*-chloromercuribenzoate in modifying the spectrum of the reduced enzyme (especially that portion which has been ascribed to the formation of flavin semiquinones), suggest that the FAD exists in this state in the reduced enzyme.

7. GSSG may be bound on a secondary site on the oxidized enzyme, and the GSSG so bound can react with the active sulphhydryl group produced when the oxidized enzyme is reduced with NADPH. Many salts compete competitively with GSSG for this site, and this is one of the causes for the inhibition of the enzyme by these salts.

8. Sodium iodide also inhibits by a specific effect on the sulphhydryl group on the active centre of the reduced enzyme. The evidence suggests that this group is oxidized to a disulphide. There is no evidence, as yet, to indicate whether this bond is formed between sulphhydryl groups on the same molecule, or between those on adjacent molecules.

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