The Effect of Glutathione on Protein Sulphydryl Groups in Rat-Liver Homogenates

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Barron (1951) first suggested that enzymes requiring sulphydryl groups for their activity might be protected from oxidative inactivation in the tissues by the presence of GSH, as part of a metabolic control mechanism.

This is plausible because GSH has an 'euphoristic' effect *in vitro* (Racker, 1954) on the activity of SH-enzymes and such enzymes can be inactivated by oxygen (Dickens, 1946), but adequate evidence to support or refute the theory is difficult to obtain (Boyer, 1959).

However, since enzymes are proteins, a preliminary requirement is that a part at least of the tissue protein SH should be oxidizable at 37° under normal partial pressures of oxygen and that GSH, in amounts comparable with those present in the tissues, should prevent or reverse this process.

The only tissue so far studied *in vitro* from this point of view seems to be the bovine lens, where Kinoshita & Merola (1957) found that the protein SH of homogenates was only slightly oxidized by oxygen, and added GSH not only failed to prevent this oxidation but was itself much more rapidly oxidized.

This paper reports data obtained with rat-liver homogenates, which show that in this instance the oxidation of protein SH by oxygen and its prevention by GSH under the conditions specified above can occur.

The results have been obtained by a new method for estimating protein SH in the presence of GSH.

MATERIALS AND METHODS

Bovine serum albumin was obtained from Armour Ltd. (Eastbourne, Sussex), NADP from Sigma Co. (St Louis, Mo., U.S.A.), GSH, GSSG and glucose 6-phosphate (disodium salt) from L. Light and Co. Ltd. (Colnbrook, Bucks.), EDTA from British Drug Houses Ltd. (Poole, Dorset) and di-(5-carboxy-4-nitrophenyl) disulphide from Aldrich Co. (Wis., U.S.A.). GSH was standardized by iodate titration (Woodward & Fry, 1932). 'Phosphate saline' was made up as described by Pinto (1961). Phosphate buffers (pH 6·0, pH 6·8 and pH 7·6) were made up as described by Datta & Grzybowski (1961) at *I* 0·2 ('strong') and 0·05 ('weak').

Preparation of homogenates. Albino Norway rats were used. Liver, removed immediately after death, was cooled in ice-cold KCl (0·9%), roughly weighed and homogenized at 0° in a Potter–Elvehjem-type homogenizer with 'phosphate saline'. The volume was adjusted to give a 20% (w/v) homogenate and centrifuged at 5° for 10 min. at 2000*g*. The supernatant homogenate was used within 1 hr. of death. Dialysed homogenates were obtained by dialysis for 16 hr. at 5° against phosphate saline (at least 100 vol.).

Incubations. Homogenates were shaken in a thermostat and sampled at intervals. Automatic analysis of non-protein SH and total SH (see below) was begun within 10 sec. of sampling. Evacuated Thunberg tubes were used for the anaerobic experiments.

Manual estimation of sulphydryl groups. Ellman (1959) found that the aromatic disulphide, di-(5-carboxy-4-nitrophenyl) disulphide, is reduced at pH 8 by non-protein SH to the corresponding aromatic thiol compound. This substance, unlike the original disulphide, has an intense yellow colour and its concentration can therefore be estimated spectrophotometrically. In the present work a Unicam spectrophotometer (SP. 500) was used. Solutions containing GSH (up to 0·3 μmole), 10% homogenate (0·2 ml.) or bovine serum albumin (up to 30 mg.) were made up to 1 ml. with phosphate saline and additions made as shown below.

Non-protein sulphydryl groups. Weak phosphate buffer, pH 6·8 (1·5 ml.), and the disulphide (mM in strong phosphate buffer, pH 6·8; 0·5 ml.).

Total sulphydryl groups. Weak phosphate buffer, pH 7·6 (1·5 ml.), and the disulphide (10 mM in strong buffer, pH 7·6; 0·5 ml.).

The solutions were stirred and the extinctions at 412 mμ in a 1 cm. cell were determined 1–6 min. later (see Results section) for non-protein SH and 15–100 min. later for total SH. Absorption due to colour or turbidity in the protein solutions was subtracted. This was determined in a separate sample (1 ml.) to which buffer (2 ml.) had been added.

Automatic estimation of sulphydryl groups. An Auto-Analyzer (Technicon Ltd., London) was used to estimate solutions containing up to 2 μmoles of total SH/ml. at the rate of 20/hr. These solutions, alternated with water, were subjected to the following mechanical operations (see Fig. 1).

Non-protein sulphydryl groups. Each sample was aspirated at a flow rate of 0·42 ml./min., mixed with weak pH 6·0 buffer (1·2 ml./min.) and segmented with air (0·8 ml./min.). It was dialysed, a D-30 cellophan membrane (Technicon Ltd.) being used at 35° against a water stream (1·6 ml./min.) also segmented with air (0·8 ml./min.). The water, containing dialysed non-protein SH, upon leaving the dialyser unit was mixed with the disulphide (mM in strong pH 7·6 buffer; 0·2 ml./min.), when, after passing through a 2 min. delay coil, the colour developed was scanned in the colorimeter unit in a 6 mm. flow cell with

a 410 mμ filter. The extinction of the reaction mixture, plotted automatically on logarithmic paper, was subsequently converted into μmoles of non-protein SH/ml. by comparison with the values (linear with concentration) obtained with four standard solutions of GSH (0·5, 1·0, 1·5, 2·0 μmoles/ml.).

Total sulphydryl groups. The sample (0·6 ml.), which had been mixed manually with the disulphide (10 mM in strong pH 7·6 buffer containing 10 g. of EDTA/l.; 0·1 ml.) and kept at 5° for 1–1½ hr., was analysed as for non-protein SH except that weak pH 6·8 buffer was replaced by weak pH 7·6 buffer and the disulphide solution by strong pH 7·6 buffer. Values obtained by this procedure, applied to the same standard GSH solutions as used for non-protein SH, were used to convert the readings into μmoles of total SH/ml. The figures were adjusted as indicated under Results.

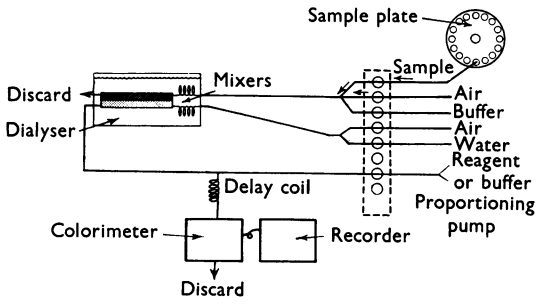

Fig. 1. Flow diagram for the automatic estimation of SH groups. The proportioning pump, sample plate with samples, water, buffer and reagent were kept in a refrigerator at 4°. For details see the text.

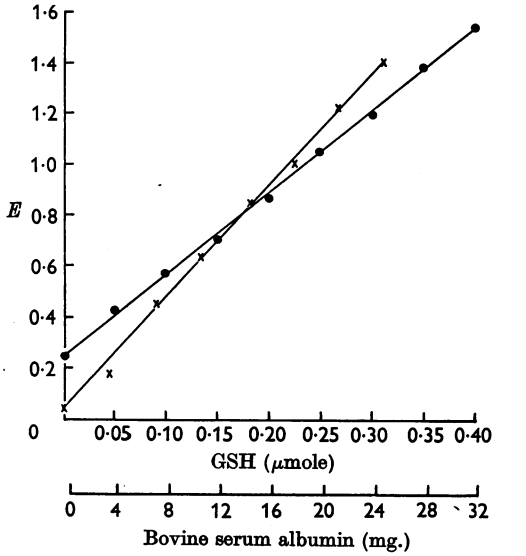

Fig. 2. Effect of varying concentrations of GSH (×) or bovine serum albumin (●) on *E* at 412 mμ after reaction with di-(5-carboxy-4-nitrophenyl) disulphide. GSH was estimated as non-protein SH by adding mM-disulphide at pH 6·8, and bovine serum albumin as total SH by adding 10 mM-disulphide at pH 7·6 1 min. and 15 min. respectively after addition of the reagents, as described for the manual estimation in the Methods section. Concentrations given are the amounts in the 1 ml. of sample estimated.

RESULTS

Ellman (1959) first used di-(5-carboxy-4-nitrophenyl) disulphide at pH 8 to estimate SH. Under his conditions GSH and cysteine react quantitatively with the reagent whereas the SH of bovine serum albumin reacts slowly and incompletely. It has now been found that, if the pH of the medium is reduced to pH 6·8, the extinction at 412 mμ developed with GSH is unchanged and linear with concentration (Fig. 2), but there is then virtually no reaction with bovine serum albumin. The extinction developed by GSH is also unchanged if at pH 7·6 the concentration of the disulphide used is increased tenfold, whereas under these conditions bovine serum albumin reacts with the disulphide, developing an extinction which is linear with concentration (Fig. 2). Calculation of the bovine serum albumin SH from the molar extinction coefficient obtained with GSH gives a value (based on a molecular weight of 66 000; Edsall, 1954) of 0·60 SH group per molecule, in reasonable agreement with published values (Kolthoff, Anastasi, Stricks, Tan & Deshmukh, 1957, give 0·68 by amperometry). This fractional SH titre is known to be due to the heterogeneity of the protein, which contains both mercaptalbumin (with one SH group per molecule) and, according to King (1961), a mixed disulphide formed between the SH of mercaptalbumin and cysteine.

The different reactivity of non-protein SH and protein SH towards the disulphide enables non-protein SH to be estimated at pH 6·8 in the presence of protein SH. Total SH (protein plus non-protein SH) can then be estimated at pH 7·6 and thus a value for protein SH found by difference. The application of this method to mixtures of bovine serum albumin and GSH is illustrated in Fig. 3.

Non-protein and protein sulphydryl groups in rat-liver homogenates. The same method has been used with 10 % rat-liver homogenates. In this case the maximum colour produced in the estimation of total SH takes longer (40 min.) to develop; it is then stable at 5° for at least a further hour. On the other hand non-protein SH must be estimated 1 min. after addition of the reagent since there is a slow further increase in extinction, presumably due to some reaction with protein SH.

If the proteins in the homogenate are precipitated with metaphosphoric acid and the pH is adjusted, no difference is then found between the estimations of non-protein and protein SH; the difference found

in the original homogenate is therefore a measure of the protein SH concentration. Non-protein SH and total SH determined in this way are both proportional to homogenate concentration when this is varied, provided that an ice-cold homogenate is used and the reagents are added immediately after sampling. Any delay leads to a loss of non-protein SH. This is shown in Table 1, which illustrates the effect of a delay (15 min.) between addition of the disulphide for the total SH estimation and addition of the disulphide for the non-protein SH estimation to different samples of the same homogenate. Recoveries of GSH added to the homogenate and estimated as non-protein SH are very poor whereas recoveries as total SH are satisfactory, though this estimation is completed much later. This difference is probably due to the rapid oxidation of GSH by the homogenate (Fig. 5), which is arrested as soon as the disulphide is added. The availability of an apparatus for analysing total SH and non-protein SH automatically (AutoAnalyzer) has enabled the latter to be conveniently estimated immediately after sampling. The technique used for non-protein SH (see Methods section) differs from the manual estimation in that a definite fraction of the non-protein SH is first dialysed from the protein solution into water and this subsequently reacts with the disulphide. For the total SH analysis a further portion of the homogenate, sampled at the same time, is added directly to the disulphide, kept for 60–90 min. at 5° and then treated essentially as for non-protein SH. In this case a fraction of the developed colour dialyses into water and this is subsequently estimated.

These methods give an increase in extinction proportional to homogenate concentration and quantitative recovery of added GSH analysed as non-protein SH is now obtained (Fig. 4). However,

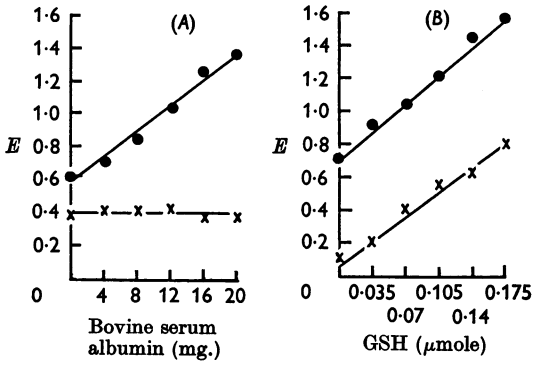

Fig. 3. Variation of E due to non-protein SH (×) and total SH (●) in mixtures of GSH and bovine serum albumin. Non-protein SH and total SH were estimated manually as described in Fig. 2, except that for non-protein SH E was read 6 min. after addition of the reagents. In (*A*) the mixture analysed contained the stated amounts of bovine serum albumin per ml. of GSH solution (0·07 μmole/ml.). In (*B*) the mixture contained the stated amounts of GSH per ml. of bovine serum albumin solution (12 mg./ml.).

Table 1. *Recovery of glutathione from rat-liver homogenates*

Ice-cold 20% homogenate (2 ml.) was mixed with phosphate saline (2 ml.) containing the GSH. Duplicate portions (0·2 ml.) were made up to 1 ml. with ice-cold phosphate saline. The buffer and reagent were added immediately for the total SH estimation and after a lapse of 15 min. for the non-protein SH estimation. Both estimations were performed manually, as described in the Methods section, 40 min. and 1 min. respectively after addition of the reagents. Values are given as μmoles of SH/ml. of 10% homogenate.

	Determination of non-protein SH		Determination of total SH	
GSH added	Non-protein SH found	GSH recovery (by diff.)	Total SH found	GSH recovery (by diff.)
Nil	0·30	—	0·90	—
0·21	0·33	0·03	1·05	0·15
0·42	0·30	0	1·29	0·39
0·63	0·31	0·01	1·46	0·57
0·84	0·33	0·03	1·76	0·86

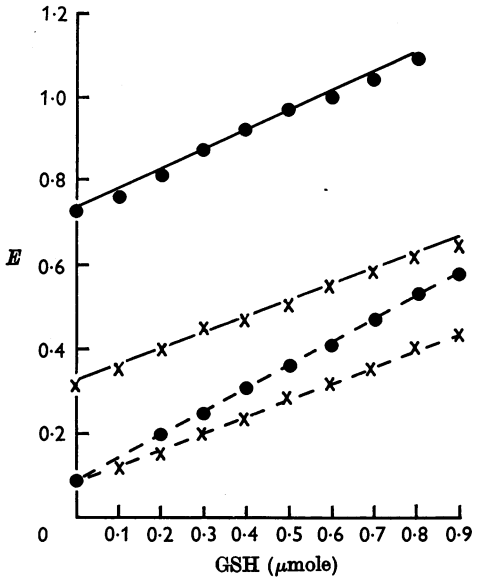

Fig. 4. Comparison of E developed by GSH when added to phosphate saline solutions (broken lines) or to 20% rat-liver homogenates (continuous lines) and estimated automatically as non-protein SH (×) and total SH (●) as described in the Methods section. The sample contained the amount of added GSH (μmole/ml.) specified. Note that the two non-protein SH lines are parallel and the total SH lines converge with increasing concentration of GSH.

GSH analysed as total SH is recovered only to the extent of about 90% at all concentrations of added GSH (Fig. 4). This is presumably due to binding to the homogenate proteins of some of the colour developed. All the values for total SH obtained with a given homogenate have therefore been adjusted (by a factor of about 1·1) so as to give 100% recovery of added GSH as total SH from ice-cold homogenates sampled immediately after mixing. From these corrected figures for total SH, protein SH has been obtained as before by subtracting the non-protein SH values.

Effect of incubation at 37° on the non-protein and protein sulphydryl groups of homogenates. Incubated homogenates with or without added GSH were sampled at intervals and the non-protein SH and protein SH concentrations were determined by automatic analysis. Typical results, obtained with different homogenates, are plotted in Fig. 5, which also specifies the conditions used for the other homogenates mentioned below. The following points are noted.

(i) Non-protein SH or added GSH was lost during aerobic incubation at the rate of 0·55 μmole (S.D. 0·23)/ml. of 10% homogenate in 30 min. (10 homogenates) (e.g. Fig. 5*A*, *B*, *C*). The loss was prevented by excluding air (Fig. 5*A'*).

(ii) Protein SH usually fell during aerobic incubation but this fall was reduced by the previous addition of GSH and to an extent depending on the amount added (Fig. 5*A*, *B*). The following data were obtained with 11 different homogenates.

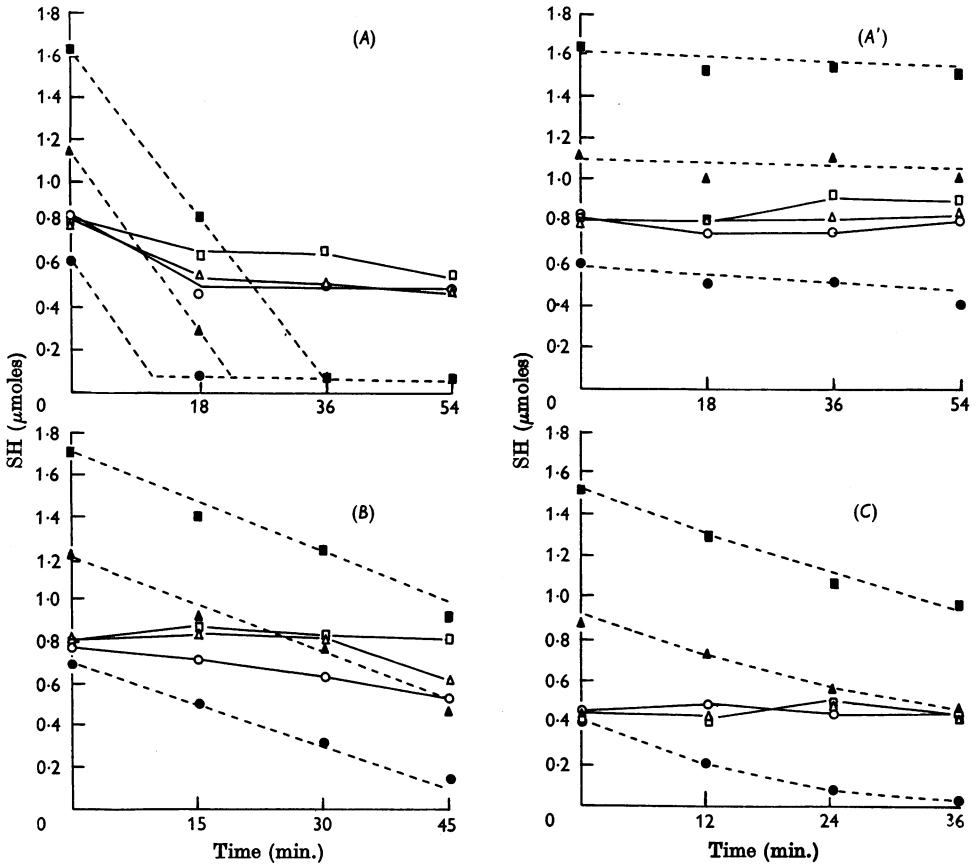

Fig. 5. Effect of aerobic (*A*, *B*, *C*) and anaerobic (*A'*) incubation at 37° on the non-protein SH (broken lines; ●, ▲, ■) and protein SH (continuous lines; ○, △, □) of rat-liver homogenates. The fresh ice-cold 20% homogenate was added to an equal volume of ice-cold phosphate saline without GSH (●, ○) or with GSH, 0·5 (▲, △) or 1 (■, □) μmole/ml. Mixtures were sampled immediately before and at intervals during incubation as indicated. Non-protein SH and protein SH were estimated automatically (see Materials and Methods section), and the results are given as μmoles of SH/ml. of 10% homogenate. Different homogenates provided the data for (*A*), (*B*) and (*C*) but the same homogenate was used for both (*A*) and (*A'*), where the aerobic and anaerobic incubations were concurrent.

Table 2. *Effect of nicotinamide–adenine dinucleotide phosphate and glucose 6-phosphate on the protein sulphydryl groups of rat-liver homogenates*

The 20% homogenate (3 ml.) was incubated anaerobically at 27° with phosphate saline (1·5 ml.) and the additions listed to give the following final concentrations (μmole/ml. of solution): GSSG (0·67), glucose 6-phosphate (0·67), NADP (0·05). Non-protein SH and protein SH were estimated automatically as described in the Methods section. Figures represent μmoles of SH/ml. of incubated solution.

	Non-protein SH			Protein SH		
Incubation time (min.)	Nil	18	36	Nil	18	36
Additions to homogenate and phosphate						
Nil	1·07	1·03	1·10	1·24	1·20	1·02
GSSG	1·10	1·37	1·46	1·15	1·13	1·04
Glucose 6-phosphate + NADP	1·07	1·09	1·10	1·16	1·08	0·93
GSSG + glucose 6-phosphate + NADP	1·06	1·38	1·53	1·20	1·17	1·07

Initial protein SH concentration, 0·78 μmole (S.D. 0·16)/ml. of 10% homogenate; final protein SH concentration (after 30–60 min. incubation): (*a*) without added GSH, 0·51 μmole (S.D. 0·15); (*b*) with added GSH (0·5 μmole/ml. of homogenate), 0·59 μmole (S.D. 0·13); (*c*) with added GSH (1·0 μmole/ml.), 0·71 μmole (S.D. 0·16). When no fall in protein SH was observed in the absence of added GSH (three homogenates, e.g. Fig. 5*C*) the initial concentration of protein SH was already low (0·33, 0·42, 0·42 μmole/ml. of 10% homogenate).

(iii) A fall in protein SH was not found during anaerobic incubation (three homogenates, e.g. Fig. 5*A'*) except in one instance where the fall (from 1·0 to 0·66 μmole/ml. of 10% homogenate in 36 min.) was less than for the same homogenate incubated aerobically (from 1·0 to 0·37 μmole/ml.). The effect of GSH on protein SH during anaerobic incubation was variable. There was either no difference in the protein SH value as a result of adding GSH (two homogenates), a significant increase (one homogenate, Fig. 5*A'*) or prevention of the anaerobic fall in protein SH (one homogenate).

(iv) The initial value for protein SH found after dialysing homogenates was low. The results obtained with two homogenates were: before dialysis, 0·60 and 0·72 μmole/ml. of 10% homogenate; after dialysis, 0·49 and 0·30 μmole/ml., respectively. Subsequent incubation of these dialysed homogenates with or without added GSH produced no further change in their protein SH values.

Effect of NADP and glucose 6-phosphate on the protein sulphydryl groups of homogenates. Enzymes utilizing $NADPH_2$ to reduce protein disulphide groups to protein SH have been described (Black, Harte, Hudson & Wartofsky, 1960; Hatch & Turner, 1960) and so the effect of NADP and glucose 6-phosphate on the protein SH of homogenates was studied.

No significant difference could be found in the protein SH (six homogenates) after aerobic or anaerobic incubation at 37° under the conditions described in Fig. 5, with or without previous addition of glucose 6-phosphate and NADP (final concentrations, 2 μmoles and 0·05 μmole respectively/ml. of 10% homogenate). However, no reduction of added GSSG (final concentration, 0·5 μmole/ml.) was observed either, so that in these experiments there was no guarantee that endogenous glucose 6-phosphate dehydrogenase was active enough to produce $NADPH_2$ from NADP and glucose 6-phosphate. The reduction of GSSG in rat-liver homogenates with a lower incubation temperature (27°) and stronger homogenate was observed by Pinto (1961). Under these conditions (Table 2), GSSG when added was indeed reduced but there was still no significant effect on the protein SH of the homogenate.

DISCUSSION

Ellman (1959) showed that proteins would react with di-(5-carboxy-4-nitrophenyl) disulphide and Black *et al.* (1960) have since estimated the SH of insulin peptides with this reagent, but it has not previously been used to estimate protein SH.

With bovine serum albumin there appears to be nearly complete reaction with the available SH despite the fact that these groups are not reactive enough to be oxidized even by ferricyanide (Kolthoff & Anastasi, 1958). The reaction with rat-liver homogenates is also linear with increasing protein concentration and the mean of the protein SH values (7·8 μmoles/g. of liver) is comparable with figures obtained by amperometry (Register, La Sorsa, Katsuyama & Smith, 1959, give 9·2 and 9·8 μmoles/g. of liver for two groups of female Sprague–Dawley rats on a stock diet). Thus in this case, too, at least a substantial and constant proportion of the protein SH present reacts with the disulphide.

Klotz, Ayers, Ho, Horowitz & Heiney (1958) showed that when di[2-(3-hydroxy-6-sulphonaphth-

1-ylazo)phenyl] disulphide reacted with bovine serum albumin, the sulphydryl compound first formed was oxidized by the protein disulphide bonds to form a mixed disulphide. Though a similar reaction apparently does not occur between bovine serum albumin and di(5-carboxy-4-nitrophenyl) disulphide, such a reaction with liver-homogenate proteins might account for the finding that a small fraction (about 10%) of the colour produced is non-diffusible. The method applied to homogenates clearly shows that a substantial fraction (about a third) of their protein SH is rapidly autoxidizable (since no fall normally occurs in the absence of oxygen) and that this is decreased or prevented by GSH. GSH is itself also rapidly oxidized by the homogenate but the rate of this oxidation is not dependent on its initial concentration. Contrariwise, the protective effect on protein SH increases with GSH concentration. This would seem to show that the SH of GSH is preferentially oxidized over that of protein SH rather than that GSH functions by continually converting the oxidized form (presumably a disulphide) of protein SH back into its reduced state. This view is also supported by the fact that, when protein SH is lowered as a result of dialysis, GSH will not subsequently restore its concentration again. $NADPH_2$ will not elevate protein SH values in homogenates either and there is therefore no evidence for a $NADPH_2$-requiring protein disulphide reductase in rat liver.

It seems then that the data now reported are compatible with a protective role for GSH on the protein SH of tissues but do not favour the idea implicit in Barron's (1951) hypothesis of an equilibrium involving the oxidized and reduced forms of the two substances.

SUMMARY

1. A method is described for estimating protein and non-protein sulphydryl groups in the presence of each other by means of the reagent di-(5-carboxy-4-nitrophenyl) disulphide. This method has been adapted for use in an apparatus for automatic analysis.

2. The method has been applied to mixtures of bovine serum albumin and glutathione and to the protein sulphydryl groups and non-protein sulphydryl groups of rat-liver homogenates.

3. Some of the protein sulphydryl groups in rat-liver homogenates are autoxidizable at 37° and this oxidation is reduced or prevented by glutathione to an extent dependent on its initial concentration.

4. Reduced nicotinamide–adenine dinucleotide phosphate does not increase protein sulphydryl groups in rat-liver homogenates.

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