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The Relationship between Glutathione and Protein Sulphydryl Groups in Germinating Pea Seeds

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After the work of Hopkins & Morgan (1943), Spragg & Yemm (1959) showed that in the early stages of germination of pea seeds there was a rapid and quantitative conversion of GSSG into GSH, and it was suggested that the production of large quantities of GSH may precede the reduction of protein disulphide groups.

The activities of many enzymes depend on the presence of free chemical groups on the protein chains. Some enzymes, e.g. the glycolytic enzymes, depend on the sulphydryl group, oxidation of which to the disulphide form inactivates the enzymes [Hopkins, Morgan & Lutwak-Mann (1938), with GSSG as oxidizing agent]. In contrast, White (1960) showed that the activity of ribonuclease was a function of the number of disulphide bonds in the protein chain, and Liener (1957) found a similar relationship for the activity of trypsin.

Results from experiments *in vitro* show that reactions can occur between protein disulphide groups and GSH; for instance, Narahara & Williams (1959) reduced the disulphide bonds of insulin with

GSH in the presence of an enzyme prepared from liver. In addition to enzymically induced reactions, Ryle & Sanger (1955) showed that an interchange occurred between disulphide groups in the presence of acid, and Huggins, Topley & Jensen (1951) suggested that a sulphydryl-disulphide interaction took place when albumins were dissolved in urea solution. This evidence suggests that the redox state of the protein sulphydryl groups can be readily affected by other thiols.

It appears possible that in a cell the balance between the activities of different classes of enzymes could be controlled, in part, by the oxidation-reduction state of the sulphydryl group alone, and the present study was made to obtain information on the reaction between glutathione and the protein sulphydryl or disulphide groups in the intact cell.

MATERIALS AND METHODS

Plant materials. Pea seeds (var. Meteor) were used. They were surface-sterilized with 3% (w/v) calcium hypochlorite, and were washed with sterile water before germination was started. This was taken as the start of an experiment and all times were measured from this point. The seeds were germinated in an aerated solution or in

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water, and if the experiment lasted longer than 24 hr. the soaked seeds were sown in soil and the seedlings grown in warmed glass-houses. The water-treated seeds gave 98–100% germination. In the experiments with *N*-ethylmaleimide the percentage germination after 10 days was assessed on 100 seeds.

Chemicals. *N*-Ethylmaleimide and EDTA (disodium salt) were obtained from L. Light and Co., Colnbrook, Bucks. AnalaR grades of potassium chloride, ammonium sulphate and sodium phosphates were used.

Extraction and estimation of oxidized and reduced glutathione. The procedures used were those described by Spragg & Yemm (1959).

Extraction of proteins. All operations were carried out below 4°. The proteins were extracted with buffer containing: 0.05M-disodium hydrogen phosphate; 0.2M-potassium chloride; 1.0 mM-EDTA, adjusted with sodium hydroxide to pH 7.3. (EDTA was added to prevent free metal ions from catalysing the oxidation of the thiol groups during the extraction.) For analysis of dry seeds, these were ground to a fine powder before extraction. Plant material (10 g.) was ground to a paste with buffer (120 ml.); after 5 min. the macerate was centrifuged at 1600g for 10 min. The supernatant liquid was stored while two further extracts of the residue were made, and the extracts were combined for analysis. This procedure gave almost complete extraction of the nitrogenous compounds soluble in this solvent, i.e. about 70% of the total nitrogen from the seeds. The extractions were carried out in duplicate, and the estimates of their protein-sulphydryl contents agreed to within $\pm 10\%$ of the mean.

Removal of low-molecular-weight compounds from the extract. The extraction procedure was unselective, and glutathione was present in the extract. Two methods were tested, for removing this compound. In the first, the solution was 75% saturated with ammonium sulphate and, after centrifuging at 20 000g, the precipitated proteins were washed with water and redissolved in buffer; in the second, the extract was dialysed at 4° overnight, and this was found to be a sufficient time to remove all the glutathione.

The two methods did not give comparable results, and the protein-disulphide content was greatly affected by the procedure used. Consistently, more disulphide groups were detected after dialysis than after the precipitation procedure (4.5 and 3.7 as against 0.2 and 0.4 μ moles/g. dry wt., after germination for 0 and 16 hr. respectively); but, despite the possibility that changes could have occurred during dialysis, this procedure was adopted.

Estimation of the protein sulphydryl groups. The protein sulphydryl groups were estimated by the amperometric titration method described by Benesch, Lardy & Benesch (1955) with 1.0 mM-silver nitrate. In view of the difficulties which we and other workers have encountered with the rotating platinum electrode (see Burton, 1958; Sluyterman, 1957), our technique for treating the electrode before titrating the thiols is described in detail. The platinum wire was sealed in soda-glass tubing and the contact made with the external circuit via mercury in the tube. The electrode was first coated with mercury by rotating it in 1.0% (w/v) mercuric chloride in 0.5N-ammonia. The plating was facilitated by the p.d. existing between the electrode and the reference cell, and 30 min. was sufficient time for an adequate layer to form. The electrode was then

washed by rotating it in several changes of water until the moles of GSH (used as the reference compound) titrated and the g.ions of Ag^+ ions added were equivalent, and then washed finally in 0.01% cysteine hydrochloride.

Reduction of protein disulphide groups. Two methods were compared: (a) the sodium tetrahydroborate (NaBH_4) method of Moore, Cole, Gundlach & Stein (1958), and (b) the milder procedure, with sodium sulphite, described by Carter (1959). Bailey & Cole (1959) showed that the ease with which the disulphide bond could be reduced varied with the protein species, and, therefore, the optimum times for reduction of the pea-seed proteins were determined. With the sodium tetrahydroborate method, incubation for 40 min. at 37° gave the greatest titre; after this time the titre decreased, the decrease not being prevented by the addition of more sodium tetrahydroborate. With sodium sulphite, reduction was complete a few seconds after adding the reagent at 37°, and remained relatively constant for up to 10 min. In both methods urea (final concn. 8M) was added to the reducing mixtures. After reduction with sodium tetrahydroborate, estimates of disulphide groups were significantly higher than after reduction with sodium sulphite (0.25, s.d. ± 0.02 , and 0.18, s.d. ± 0.031 , as against 0.00 and 0.07 μ mole/mg. of non-diffusible nitrogen, after germination for 0 and 16 hr. respectively). The highly alkaline conditions of the sodium tetrahydroborate method caused some protein breakdown and ethanol-soluble peptides were present in the reaction mixtures after incubation. A check on the tetrahydroborate method was made by adding the total sulphydryl sulphur (determined after tetrahydroborate reduction) to the methionine-sulphur content of the proteins from dry seeds, calculated from previous data (Spragg, 1955); the total was found to agree, within the limits of experimental error, with the estimate for the total sulphur of the proteins. Therefore the sodium tetrahydroborate method was used for the reduction of the protein disulphide bonds.

Estimation of total nitrogen. The semimicro-Kjeldahl procedure was used and analyses were made in duplicate.

Estimation of total sulphur. Duplicate extracts of the proteins were made from 30–40 g. dry wt. of seeds and, after dialysis, the solution of non-diffusible compounds was evaporated to dryness in a porcelain crucible. The total sulphur in each residue was estimated in duplicate by the standard macromethod (Association of Official Agricultural Chemists, 1950). The results for the duplicate extracts agreed to within $\pm 2\%$ of the mean.

Dry-weight contents. These were determined by drying the materials to constant weight at 104°.

RESULTS

Relationship between glutathione content and germination

Attempts were made to influence the change from GSSG to GSH by the addition of inhibitors known to react preferentially with the sulphydryl group. Roberts & Rouser (1958) noted that *N*-ethylmaleimide reacted more slowly with the protein sulphydryl group than with GSH, and this compound proved the most useful in the present work. Table 1 shows that, in seeds soaked in

Table 1. *Effect of N-ethylmaleimide on the glutathione content of intact seeds*

The seeds were soaked for 24 hr. in either 10 mM- or 5 mM-*N*-ethylmaleimide, and were then transferred to water. Samples of seed were taken after the periods shown; these periods were measured from the time the seeds were first placed in the *N*-ethylmaleimide solutions.

Period of seed germination (hr.) ...	Concn. of <i>N</i> -ethylmaleimide (mM)	Sulphydryl and disulphide groups (μ moles/g. dry wt.)				
		8	16	24	42	90
GSH	0	1.20	2.00	—	2.40	2.80
	10	0.12	0.20	—	0.16	0.12
GSSG	0	0.52	0.40	—	0.32	1.04
	10	0.84	0.48	—	0.48	0.44
GSH + GSSG	0	1.72	2.40	—	2.72	3.84
	10	0.96	0.68	—	0.64	0.56
GSH	0	2.60	2.44	2.72	3.12	2.28
	5	1.32	1.04	2.04	3.12	1.68
GSSG	0	0.44	0.32	0.32	0.44	0.44
	5	0.44	0.04	0.24	0.56	0.44
GSH + GSSG	0	3.04	2.76	3.04	3.56	2.72
	5	1.76	1.08	2.28	3.68	2.12

10 mM-*N*-ethylmaleimide, the total glutathione content was decreased and no net synthesis occurred even after 90 hr. A concentration of 5 mM-*N*-ethylmaleimide caused an initial decrease in GSH, but after 42 hr. the concentrations of both the total glutathione and the GSH were similar for the treated and the untreated seeds.

The germination of the pea seed appears to depend on the GSH content of the seed, and the linear relationship between GSH concentration and the percentage germination of the seed population is shown in Fig. 1.

Changes in the sulphydryl and disulphide content of protein during germination

The sulphydryl and disulphide contents of the seed proteins were estimated at several stages of germination. *N*-Ethylmaleimide selectively removed GSH from the seeds, and, when the external concentration of *N*-ethylmaleimide was less than the concentration of the GSH in the soaked seeds, the total determined protein-(sulphydryl + disulphide) concentration was not affected but the sulphydryl:disulphide ratio decreased.

There was a decrease in both the sulphydryl and disulphide content of the proteins during this period. This decrease could not be explained by breakdown of the proteins to diffusible products during the first 16 hr. of soaking since the amount of diffusible nitrogen remained at 2.1 mg./g. dry wt. Further, no evidence could be found for any loss of the total sulphur from the proteins during this period.

Fig. 1. Relationship between the GSH content of the seed and the percentage germination of the seed population.

Equilibrium between the sulphydryl and disulphide groups of the protein

Seeds were soaked in different concentrations of *N*-ethylmaleimide and the protein sulphydryl and disulphide groups were estimated after 16 hr. Table 2 shows that, except for seeds which had been soaked in the highest concentration of *N*-ethylmaleimide, the determinable (sulphydryl + disulphide) content was not different from that of seeds which had been soaked in water. The sulphydryl:disulphide ratio, however, was affected by the *N*-ethylmaleimide treatment, and decreased with increasing *N*-ethylmaleimide concentration. The linear relationship between the percentage germination of the seeds and the sulphydryl:disulphide ratio is shown in Fig. 2.

Table 2. *Changes in the sulphydryl and disulphide content of the proteins of intact seeds after soaking in different concentrations of N-ethylmaleimide for 16 hr.*

s.e. values of a single estimate were: for the water-treated seeds, $\pm 0.6 \mu\text{mole}$ of sulphydryl/g. dry wt. and $\pm 1.0 \mu\text{mole}$ of disulphide/g. dry wt. Results are expressed as $\mu\text{moles/g. dry wt.}$

Concn. of <i>N</i> -ethyl- maleimide (mM)	SH	S·S	SH + S·S
10	1.3	6.6	7.9
8	2.5	7.0	9.5
6	3.4	6.0	9.4
4	4.6	5.4	10.0
2	4.9	5.8	10.7
1	4.9	4.4	9.3
0	4.7	5.8	10.5

disulphide, 0.24 ± 0.04 ; total, 0.42 ± 0.04 . After germination for 16 hr. the values were: sulphydryl, 0.14 ± 0.02 ; disulphide, 0.18 ± 0.02 ; total, 0.32 ± 0.04 . All the estimates were made in the presence of urea. The possibility that removal of some of the determinable protein sulphydryl groups was masked by a continual replenishment from the non-determinable (sulphydryl + disulphide) pool cannot be excluded by the present experiments. However, the systematic change of the sulphydryl:disulphide ratio with changes in the *N*-ethylmaleimide concentration, the results of Roberts & Rouser (1958), and the similar (sulphydryl + disulphide) concentration in both the treated and the untreated seeds, suggest that this type of interchange was of minor importance in these experiments.

Changes in the sulphydryl and disulphide of proteins and of glutathione during germination could be explained by the existence in seeds of an equilibrium of the type:

For example, if GSSG increases, the protein disulphide must also increase at the expense of the protein sulphydryl groups. Such a process would increase the activity of enzymes of the ribonuclease (White, 1960) and trypsin type (Liener, 1957) in the cell. Conversely, an increase in GSH as in the germinating seed must be accompanied by a proportional increase in the protein sulphydryl groups, so increasing the activity of the glycolytic and other respiratory enzymes. Changes such as these could explain the dependence of germination on the redox state of the sulphydryl groups.

Hatch & Turner (1959) have been able to simulate the Pasteur effect in pea-seed extracts by increasing the protein sulphydryl groups in anaerobic conditions. Similarly, Rapaport & Scheuch (1960) have related the stability of pyrophosphatase in the reticulocyte with the presence of GSH, and Mazia (1954) and Hughes & Spragg (1958) have stressed the importance of reactions between glutathione and sulphydryl groups in mitotic division. Further, these types of reactions may result in the morphological effects discussed by Brachet (1959).

SUMMARY

1. The soaking of pea seeds in increasing concentrations of *N*-ethylmaleimide produced a proportional decrease in the GSH concentration in the seed. This in turn was found to decrease the sulphydryl:disulphide ratio of the proteins without changing the determinable protein (sulphydryl + disulphide) concentration. These treatments markedly decreased the percentage germination of the seeds.

Fig. 2. Relationship between the sulphydryl:disulphide ratio of the proteins and the percentage germination of the seed population.

DISCUSSION

During germination of pea seeds there was a decrease in the amount of determinable protein disulphide groups as determined by the tetrahydroborate method; good correlations have been established between the percentage germination of the seeds and (a) the concentration of GSH, and (b) the protein sulphydryl:disulphide ratio in the seeds.

Practical difficulties made it impossible to determine directly whether the protein sulphydryl:disulphide ratio altered during germination. However, the results obtained using *N*-ethylmaleimide showed that the two redox states of the protein sulphydryl group were maintained at a steady-state equilibrium by an active process involving GSH. Before soaking, the protein sulphydryl and disulphide and total (sulphydryl + disulphide) values ($\mu\text{moles/mg. of non-diffusible nitrogen}$) with s.d. values were: sulphydryl, 0.18 ± 0.01 ;

2. The number of protein disulphide groups reduced was greater with sodium tetrahydroborate than with sodium sulphite. In an extract of seeds which had been soaked for 16 hr., sodium tetrahydroborate did not reduce all the disulphide groups.

3. The results suggest that chemical reactions take place *in vivo* between glutathione and protein sulphydryl groups.

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Porphyrins from Congenitally Porphyric (Pink-Tooth) Cattle

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Of all types of porphyria, congenital or erythropoietic porphyria is the most rare. Very few cases have been reported. Information on the excretion of porphyrins is meagre and sometimes even confused by results from cutanea tarda cases (Watson, Perman, Spurrell, Hoyt & Schwartz, 1959). Ever since Fourie (1936) and Rimington (1936) reported detailed studies of a herd of affected cattle, more reports and also more controversial findings have appeared. Whereas Fourie (1936) concluded that congenital porphyria is inherited as a Mendelian recessive characteristic in cattle, Jorgensen & With (1955) reported it to be a dominant character in swine. Most investigators have agreed that porphyrins from cases of the congenital disease are mainly those of the I series. Ellis, Barner, Madden, Melcer & Orten (1958) reported a predominance of coproporphyrin III and uroporphyrin III in bovine cases, whereas Rhode & Cornelius (1958) found no uroporphyrin III in samples from porphyric heifers.

This report is concerned with biochemical features of congenital porphyria (pink tooth) of a herd of cattle in Michigan, U.S.A. Besides the well known copro- and uro-porphyrin, special attention has been paid to the isolation and characterization of the penta-, hexa- and hepta-carboxylic porphyrin from samples of urine, blood and post-mortem materials. Their properties have been compared with the corresponding porphyrins isolated from cutanea tarda cases.

MATERIALS AND METHODS

Materials. Blood and urine samples were collected from more than ten cattle, including nine (b, e, g, j, l, m, n and r) affected, one (Q) suspected to be the carrier of the disease, and several young and adult animals. Post-mortem tissues, including liver, muscle, teeth and bones, were obtained from the heifer (g). All samples were kept under refrigeration and sent to us by air. Most of them were analysed within 3 days after collection.