acid, and it seems legitimate to conclude that this property is inherent in α -hydroxy-*n*-fatty acids of very long chain length.

SUMMARY

1. The α -hydroxy-*n*-fatty acid component of yeast cerebrin has been investigated in detail.

2. The pure product obtained after alkaline saponification is optically active.

3. Under the usual conditions of crystallization from hot solvents the product separates as a gel. On very slow cooling from hot ethanol under special conditions crystalline material was obtained.

4. The crystals gave a very good X-ray photograph showing the characteristic intensity distribution of an α -hydroxy-*n*-fatty acid. The observed spacing was that of the A form of α -hydroxy-*n*-hexacosanoic acid, indicating that a small amount of homologous contaminant was present.

5. Melting-point and X-ray data for the *n*-fatty acids given on oxidation show that the cerebrin hydroxy acid is α -hydroxy-*n*-hexacosanoic acid with not more than 10% of α -hydroxy-*n*-tetra-cosanoic acid.

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The Estimation of Oxidized Glutathione

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Several methods have been published for estimating reduced glutathione (GSH) (Guthrie & Wilcoxon, 1932; Grunert & Phillips, 1951; Miller & Rockland, 1952), but no satisfactory method for estimating the oxidized form (GSSG). Hence the oxidized form can be estimated if it can be reduced quantitatively. Chemical methods for achieving this were tried (Goddard & Michaelis, 1934; Clarke, 1932; Folin & Marenzi, 1929) but were unsatisfactory quantitatively, owing to interference from phenolic compounds present in plant extracts.

Reduction of GSSG by glutathione reductase appears to be a very suitable means of estimating GSSG because the reaction is relatively rapid, the enzyme appears highly specific for GSSG, cystine and other derivatives of cystine and glutathione are not reduced (Conn & Vennesland, 1951), and the equilibrium point of the reaction between reduced triphosphopyridine nucleotide and GSSG greatly favours the formation of GSH (Mapson & Goddard, 1951; Conn & Vennesland, 1951).

Reduction of GSSG is achieved enzymically by means of malic dehydrogenase, together with glutathione reductase and triphosphopyridine nucleotide (TPN) as previously described (Mapson & Goddard, 1951). The essential reaction catalysed by glutathione reductase is

where TPNH = reduced TPN and TPN⁺ = oxidized TPN. Malic dehydrogenase enzyme with its substrate is used to regenerate reduced TPN. These enzymes are present in acetone powder preparations from pea seeds, dialysed to remove all soluble —SH and —S—S— compounds. On the addition of malate, manganese, TPN and GSSG to the dialysed extract under anaerobic conditions, hydrogen readily transfers from malate to GSSG.

Diphosphopyridine nucleotide (DPN), if present in the preparation of TPN, does not interfere in the estimation of GSSG, since the only substrate added is malate, and the malic dehydrogenase of peas is

specific for TPN (Conn, Vennesland & Kraemer, 1949). There is, therefore, no likelihood of reduced DPN being formed and of cystine being reduced by the enzyme cystine reductase, found by Nickerson & Romano (1952) to occur in yeast and in pea seeds. We have in fact shown that cystine is not reduced under the conditions in which GSSG is reduced. Krinsky & Racker (1952) reported the estimation of GSSG by yeast glutathione reductase, using DPN and alcohol dehydrogenase as the reducing system. As far as the present author is aware, glutathione reductase is specific for TPN and will not reduce GSSG with reduced DPN. In any case, as Nickerson & Romano have shown and as we confirmed, cystine if present will be reduced under these circumstances.

If the extract to be analysed contains dehydroascorbic acid the following reaction will ensue, owing to the presence of dehydroascorbic acid reductase:

The GSSG so formed will however be reduced again and, since ascorbic acid does not interfere in the estimation of GSH by the sulphur reduction method, this reaction is unimportant in the estimation of GSH, except that the time needed for complete reduction of GSSG is extended if appreciable amounts of dehydroascorbic acid are present. It is therefore advisable to carry out preliminary tests on the progress of the reaction, to determine the reaction times needed. In most tissues the concentration of dehydroascorbic acid is low, and should cause little trouble.

Cysteine if present will also be estimated, but it can be differentiated from GSH by determining (1) GSH and cysteine as here described, (2) GSH, cysteine and GSSG after reduction and (3) GSH and GSSG after first completely oxidizing all SH compounds to $-\text{S}-\text{S}-$. GSSG is measured by (2-1), and subtracting this from (3) gives GSH. This complication of the procedure is only necessary, however, when there are significant amounts of cysteine present. In preliminary experiments various oxidizing reagents were used to effect (3) (iodine, ferricyanide and 2:6-dichlorophenolindophenol), bearing in mind the necessity to avoid adding any substance which would interfere in the subsequent enzymic reduction. None proved entirely satisfactory and finally the oxidation was effected enzymically. We made use of a lipoxidase enzyme present in our enzyme preparation, which with fatty acid substrate oxidizes both cysteine and GSH to the corresponding disulphide in the presence of oxygen (Moustafa & Mapson, 1953). It is necessary to add a small amount of an activator such as caprylic alcohol, in order to bring about the break-

down of a fatty acid complex. The release of the fatty acid by the action of the alcohol provides the substrate for the lipoxidase, and this is followed by the indirect oxidation of the sulphhydryl compounds.

In this work GSH and GSSG after reduction have been estimated with minor modifications by the sulphur reduction method (Guthrie & Wilcoxon, 1932; Guthrie, 1937).

EXPERIMENTAL

Reagents

Potassium phosphate. A 40% (w/v) solution of K_2HPO_4 , freed of O_2 by storing under N_2 and flushing with N_2 before use.

The sulphur, zinc acetate, *p*-aminodimethylaniline hydrochloride and ferric chloride reagents for the estimation of GSH were made up and used as described by Guthrie (1937).

DL-Malate. This was prepared from a commercial sample of malic acid (Hopkin and Williams), made up as a solution in water in a concentration of 0.3M after neutralization to pH 6.8 with NaOH.

Manganous chloride. 0.4M solution of MnCl_2 .

Reduced glutathione. GSH prepared from yeast following the method of Pirie (1930) or as purchased commercially, is frequently contaminated with metals. Hence when it is dissolved in metaphosphoric acid solution loss of GSH occurs by oxidation. We have found it essential to remove such impurities by treating a solution of glutathione with kaolin, and removing the kaolin by centrifugation (Meldrum & Dixon, 1930).

Oxidized glutathione. GSH was dissolved in the required amount of water, and oxidized with the theoretical amount of I_2 .

Triphosphopyridine nucleotide. Highly purified preparations of TPN are not essential for the estimation of GSSG. Generally the coenzyme was prepared from horse liver by the method of Warburg, Christian & Griesse (1935), proceeding only as far as the acetone powder stage, and without purifying further on resin columns. The powder was stored at -20° , and lost no activity over long periods. More highly purified preparations, made by the method of Warburg *et al.* (1935), as modified by Haas (unpublished), or by that of LePage & Mueller (1949), have been used without any significant effect on the results. The concentration of TPN in the preparations was determined by measuring the increase in absorption in a Hilger spectrophotometer at 340 μ , after reduction by isocitrate and isocitric dehydrogenase prepared from heart muscle (Grafflin & Ochoa, 1950).

Enzyme for the reduction of GSSG (malic dehydrogenase and glutathione reductase). Dry ungerminated peas, variety Laxton Superb, were ground in an end-runner mill to a powder which was sieved through a no. 40 sieve. This powder was then extracted rapidly with 6 times its weight of cold acetone. The acetone was filtered off rapidly, and the powder spread out to dry. This kept its activity for a long time.

The above powder (20 g.) was mixed with 60 ml. of 0.025M phosphate buffer pH 6.8, allowed to stand for 5 min. at room temperature, and centrifuged. The supernatant liquid was transferred to cellophan sacs and dialysed against

5 l. of 0.01 M phosphate buffer pH 6.8 at 1°. Air was bubbled through the solution to facilitate dialysis, which was continued for 48 hr. or more. The enzyme extract was then centrifuged to remove material precipitated during dialysis, and stored at 1°, but never longer than 48 hr.

Enzymic reduction of GSSG

A 30 ml. sample of the metaphosphoric acid extract of the tissue is placed in a boiling tube in a water bath at 35°, and a stream of N_2 passed to remove O_2 , frothing being prevented by adding a trace of silicone anti-foam A. After the pH of the extract has been adjusted to approximately 6.3 with 20% (w/v) NaOH, there is added 10 ml. of dialysed pea enzyme, 40 mg. of sodium malate, $MnCl_2$ to give a final concentration of 0.024 M, and 1 mg. of TPN. The total volume is then adjusted to 50 ml., after the pH has been finally adjusted to 6.3. After the reduction is complete, the extract is acidified with 3 ml. 20% (w/v) HPO_4 . The resulting protein precipitate is centrifuged off and the centrifugate taken for the estimation of GSH by the method described below.

A typical progress curve is shown in Fig. 1. In this particular experiment, in which the reduction of GSSG was followed throughout, there was good agreement between the amount of GSH formed as estimated by (1) titration with potassium acid iodate (Fujita & Numata, 1938), and (2) by sulphur reduction.

The rate of the reduction of GSSG in this system depends, among other things, on the concentration of substrates and co-factors. A concentration of malate was used high enough not to limit the rate of the reaction. To conserve supplies of TPN, experiments were made to determine the minimum amount that could be used with the enzyme preparation, to ensure a reasonably rapid reaction. Results, relating rate of reduction to concentration of TPN, are shown in Fig. 2. In these experiments Mn was added in amount found by earlier experiments to produce an optimum effect. TPN at a concentration of 20 $\mu g./ml.$ reaction mixture is near the minimum necessary. Under these conditions we have been able to reduce 160 $\mu g.$ of GSSG/ml. reaction mixture within 1 hr. Our experience with one plant tissue, namely potato, indicates that this concentration of GSSG is considerably higher than those normally met. Extracts from tissue containing more than 160 $\mu g./ml.$ could be dealt with by dilution before test.

Good recoveries of GSSG varying between 97 and 101% were obtained when this substance was added to potato tissue in varying amounts.

Estimation of GSH by the sulphur method

The chief difficulty is to prevent oxidation during the analysis. Guthrie advocated addition of KCN to stabilize the GSH, but we have found this to give lower and more variable results than the procedure we adopted. Moreover, a slight reduction of GSSG and cystine does occur in the presence of KCN, as Guthrie (1938) has pointed out. We have found that GSH is readily oxidized during the neutralization of the metaphosphoric acid so that it is essential (1) to remove O_2 before neutralization, and (2) to neutralize with K_2HPO_4 rather than with NaOH, when there is no need to add KCN.

With this procedure results are most accurate if the sample, to be analysed contains 1–4 mg. of GSH. When known amounts of GSH were added to slices of potato tissue, recoveries in a number of experiments varied from 97 to 99%.

Fig. 1. Reduction of GSSG with dialysed pea extract. O—O, GSH estimated by sulphur reduction method; x—x, GSH estimated by acid iodate method. Reaction mixture contained malate, 0.8 mg./ml.; TPN, 20 $\mu g./ml.$; $MnCl_2$, 0.04 M; GSSG, 160 $\mu g./ml.$

Fig. 2. The rate of reduction of GSSG by dialysed pea extract as affected by concentration of TPN. Reaction mixture contained malate, 0.8 mg./ml. with varying amounts of TPN; $MnCl_2$, 0.04 M; GSSG, 150 $\mu g./ml.$ Temp. = 35°.

Procedure for the estimation of GSH

Known quantities of GSH were dissolved in 2% (w/v) HPO_4 , and 50 ml. samples were measured into Drechsel bottles (capacity 100 ml.) together with a few drops of bromothymol blue indicator, and 1 drop of capryl alcohol to prevent frothing. After a vigorous stream of O_2 -free N_2 had been passed through the solution for 10 min., the pH was adjusted to 6.6–6.8 with K_2HPO_4 solution. The O_2 -free K_2HPO_4 solution was introduced into the Drechsel bottle by means of a special inlet tube, thus excluding O_2 during the neutralization. 2 ml. of the saturated sulphur solution were added in a similar manner and the bottles connected to absorption tubes containing 25 ml. of the zinc acetate solution. Throughout these operations, and for a further period of 4 hr., a continuous stream of N_2 was passed through the Drechsel bottle. The Drechsel bottles were placed in a

water bath to maintain a temperature of 30° throughout. After 4 hr., the methylene blue developed after the addition of the necessary reagents was estimated in a Hilger Spekker photometer.

The use of lipoxidase for the oxidation of GSH and cysteine

The lipoxidase system is present in the enzyme preparation described in this paper, so it is only necessary to add the solution to be analysed to the enzyme solution after both have been adjusted to a pH of 6.3–6.5, to add a few drops of caprylic alcohol and finally to aerate the solution. The course of the oxidation of the —SH compounds may then be followed by removal of samples and titration with potassium acid iodate. 1 ml. of our enzyme preparation contains sufficient fatty acid substrate to oxidize approximately 0.3 mg. GSH or its equivalent. Larger amounts of GSH may be oxidized by the addition of small amounts of either linolenic or linoleic acid. When oxidation is complete, the air stream is replaced by O₂-free N₂ and the solution freed of O₂. The addition of malate, MnCl₂ and TPN then completes the reduction system for the conversion of GSSG to GSH. It is important that these additions should be made only after the lipoxidase activity of the extract has been arrested by the exclusion of O₂, for unless this is done a marked decrease in the activity of the reducing system may occur, and this appears to be associated with the destruction of TPN by the lipoxidase. After reduction the solution is then acidified with metaphosphoric acid and its GSH content determined by the method described above.

Using this modification of the general method, we have found that GSH may be estimated when present with cysteine with an accuracy of $\pm 5\%$ (Table 1).

Table 1. *The estimation of reduced glutathione when present with cysteine in different mixtures*

Mixture analysed		GSH found as % of that added
GSH (%)	Cysteine (%)	
75	25	95
66	34	94
25	75	96
33	67	104
50	50	104

SUMMARY

1. Oxidized glutathione may be estimated as reduced glutathione by the sulphur reduction method after enzymic reduction with triphosphopyridine nucleotide and glutathione reductase.

2. Reduced glutathione may be estimated in the presence of cysteine by first oxidizing reduced glutathione compounds enzymically by means of a lipoxidase system, followed by enzymic reduction of oxidized glutathione.

3. The method has been used with potato tissue and satisfactory recoveries of both reduced and oxidized glutathione obtained.

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