as far as possible. The precautions described above have been found to be necessary in order to achieve this.

It was thought at first that a certain amount of hydrogen chloride, as well as carbon dioxide, would diffuse into the alkali. Accordingly, 10N-phosphoric acid was tried instead of hydrochloric acid, but this did not liberate all the carbon dioxide, even after heating for 24 hr. at 150°. Sulphuric and benzenesulphonic acids were also tried, but both gave high and variable yields, probably through their acting as oxidants. Sodium hydroxide was tried in the buckets instead of barium hydroxide, the carbon dioxide being estimated by the difference of titres with acid to the phenolphthalein and methyl orange end points. Finally, by use of the titrimetric method of Sendroy (1937) to estimate chloride, it was found that no detectable amount of hydrochloric acid was reaching the barium hydroxide.

Diffusion for periods greater than 2 hr. was found to be undesirable, because of a gradual increase of the blank titre. In confirmation of the work of Tracey (1948), 5 hr. heating with acid was found to liberate all the carbon dioxide from citrus pectin, acetone glucurone and from hyaluronic acid. The yield from methyl galacturonide was incomplete in this period.

SUMMARY

A modification of the method of Tracey (1948) is described, by which uronic acids may be estimated in amounts of the order of 0·1 mg. The carbon dioxide evolved by heating with hydrochloric acid is collected in barium hydroxide and estimated by titration with acid.

We are grateful to Dr F. B. Strauss for advice; to Dr M. V. Tracey for the gift of a sample of citrus pectin; to Dr P. W. Kent for samples of acetone glucurone and of methyl galacturonide; and to the Medical Research Council for a Research Grant under which this work was carried out.

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The Reduction of Glutathione by Plant Tissues

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The close association between glutathione (GSSG) and ascorbic acid (AA) in plant tissues is well known. In germinating seeds and sprouting potato tubers ascorbic acid and glutathione appear at the same time (Pett, 1936; Hopkins & Morgan, 1943). Moreover, many plant tissues contain the enzyme dehydroascorbic acid reductase which, as Hopkins and his collaborators have shown, catalyses the transfer of hydrogen from reduced glutathione (GSH) to dehydroascorbic acid (DHA) (Hopkins & Morgan, 1936; Crook & Hopkins, 1938; Crook, 1941).

The presence of oxidative enzyme systems catalysing the oxidation of AA, such as ascorbic oxidase, polyphenol oxidase, or peroxidase, now well established, would indicate the possibility of hydrogen transfer from GSH to oxygen via the ascorbic-dehydroascorbic acid system in accordance with the following scheme:

$$2GSH + DHA \rightarrow AA + GSSG$$
$$AA + O_2 \rightarrow DHA + H_2O.$$

How far such a system represents part of a hydrogen transfer system analogous to that of the cytochrome system depends on the ability of plant tissues to reduce GSSG. That plant tissues do possess this power is indicated by the work of Kohman & Sanborn (1937) and Ganapathy (1938), who found that compounds containing —S—S— groups could be reduced by plant juice. Even more convincing was the work which has been carried out on seeds during the initial stages of germination. Firket & Comhaire (1929) noted that, while absent in

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dry pea seeds, sulphydryl compounds rapidly appear after soaking the seeds in water. Vivario & Lecloux (1930) likewise confirmed the early appearance of sulphydryl compounds after hydration of the dry seed. Hopkins & Morgan (1943) showed that sulphydryl compounds reach a maximum within 4 hr. of adding water to the dry seed. From such material they were able to isolate reduced glutathione, and to show that if oxidized glutathione was added to pea powder suspended in phosphate buffer under anaerobic conditions the glutathione was rapidly reduced.

In the present paper an attempt has been made to identify the enzyme systems concerned. We have shown the presence in ungerminated peas of two dehydrogenase enzymes each of which is capable, in the presence of coenzyme II (Co II) and metallic catalysts such as manganese or magnesium, of transferring hydrogen from its substrate to GSSG.

The presence of an enzyme in pea seeds capable of catalysing a reaction between reduced Co II and GSSG has also been shown. In a private communication, Conn & Vennesland have informed us that they also found a similar enzyme in wheat. A preliminary account of their work and of our own has already been published (Mapson & Goddard, 1951; Conn & Vennesland, 1951).

METHODS

Acetone powder from powdered ungerminated peas. Dry ungerminated peas, variety Laxton Superb, were ground in an end-runner mill (porcelain pestle and mortar) to a powder which was sieved through a no. 40 sieve. This powder was then extracted rapidly with six times its weight of cold acetone. The acetone was removed rapidly by filtration, and the powder spread out in a thin layer on paper to remove acetone. The product, a fine powder, could be kept for a long time without loss of activity.

Dialysed enzyme. The above powder (20 g.) was mixed with 100 ml. of 0·025 M-phosphate buffer, pH 6·2, and allowed to stand 5 min. at room temperature. The mixture was centrifuged, and the supernatant liquid transferred to cellophan sacs and dialysed against 4 l. of 0·025 M-phosphate buffer at +1°. Air was bubbled through the external solution to facilitate dialysis. Dialysis was usually continued for 44–48 hr. The enzyme extract was then centrifuged to remove insoluble material precipitated during dialysis. The enzyme solution contained 0·6–0·7 g. dry solids/100 ml. Glass-distilled water was used for making the buffer solutions.

Determination of enzymic activity. The dialysed or undialysed enzyme preparation (20 ml.) was placed in a tube through which N_2 was bubbled. To this solution oxidized GSSG was added together with other substances tested and water to give a final volume of 40 ml. All experiments were done at 25°. A portion of the mixture (5 ml.) was removed at appropriate intervals and pipetted directly into 25 ml. of 4% (w/v) HPO_3, water was added to give a total volume of 50 ml. and the solution centrifuged to remove the precipitated protein. The clear supernatant was then taken for the estimation of GSH.

Oxidized glutathione. A solution of GSSG was prepared by dissolving GSH (Light and Co., Colnbrook, Bucks) in the required amount of water and oxidizing the sulphydryl group by the addition of the theoretical amount of I_2. There was no evidence of any significant impurity in the GSH used. On analysis by paper chromatography it behaved as a single substance. Solutions of GSSG were freshly prepared before each experiment. The solution was then neutralized to pH 6·3 with KOH before addition to the enzyme solution.

D-iso*Citric acid.* This was prepared from the trihydrazide of the acid, by hydrolysis with H_2SO_4 and removal of the hydrazine sulphate.

DL-*Malic acid.* This was prepared from a commercial sample recrystallized three times from water.

Coenzyme II. Two samples of Co II were used. The first sample was prepared from horse liver by a method similar to that used by Warburg, Christian & Griese (1935), for its preparation from blood. This sample was of 50% purity as determined by the increase in absorption at 340 mμ. after reduction by an *iso*citric dehydrogenase enzyme prepared from heart muscle (Grafflin & Ochoa, 1950). The second sample, prepared by the method of LePage & Mueller (1949) from horse liver, was 20% pure when determined by this method.

Reduced coenzyme II. This was made by reduction of Co II with the theoretical amount of *iso*citrate by means of an *iso*citric dehydrogenase enzyme prepared from heart muscle (Grafflin & Ochoa, 1950). After the reaction was complete the solution was brought to a pH of approx. 8·5 and heated at 100° for 2 min. to inactivate enzymes. Precipitated protein was removed by centrifugation, and the solution kept at −20° until required.

Coenzyme I. The sample used was a commercial sample of 66% purity as determined by absorption at 340 mμ. after reduction by $Na_2S_2O_4$.

Dehydroascorbic acid. This was prepared by oxidation with Br_2 according to the method described by Mapson & Ingram (1951).

Estimation of glutathione and ascorbic acid. GSH formed by reduction of GSSG was estimated by titration with 0·002 N-I_2 in acid solution. The validity of using I_2 titration as an index of the formation of GSH in these experiments was confirmed by the application of the nitroprusside test for —SH compounds. Solutions from enzyme extracts reducing I_2 gave very strong nitroprusside reactions, and conversely where the increase in I_2 titre was small or negligible, weak or negative nitroprusside tests resulted. When AA was also present, the total reducing titre of the solution was determined by titration with I_2, and the AA content by titration with 2:6-dichlorophenol indophenol in acid solution (Harris & Olliver, 1942). The titre due to GSH was then calculated by difference.

RESULTS

Reduction of glutathione by undialysed extracts of ungerminated peas

We have confirmed the work of Hopkins & Morgan (1943) that powder prepared from ungerminated peas will, under anaerobic conditions, reduce GSSG. From an acetone-extracted pea flour, an enzyme solution was prepared by treating 20 g. flour with 120 ml. 0·025 M-phosphate buffer, pH 6·3, centrifuging to remove starch and cell debris, and using the

resulting enzyme solution directly. If the oxygen in the solution is removed by nitrogen there is a rapid reduction of the disulphide when GSSG is added. The results of a typical experiment illustrated in Fig. 1 show that in the enzyme solution alone there was a steady production of titratable —SH groups which was still continuing after 24 hr. when the experiment was stopped. In the solutions to which GSSG was added at three different levels, there was at first a rapid reduction of the GSSG followed by a slower increase in titratable —SH groups at a rate equal to

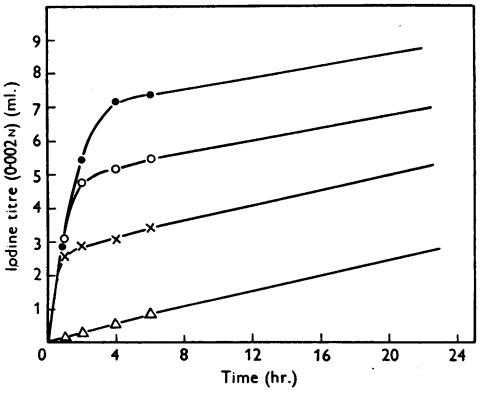

Fig. 1. Reduction of GSSG by undialysed pea extract. △—△, undialysed enzyme solution; ×—×, undialysed enzyme solution + GSSG, 0·325 mM; ○—○, undialysed enzyme solution + GSSG, 0·65 mM; ●—●, undialysed enzyme solution + GSSG, 0·975 mM. 20 ml. enzyme solution in total volume of 40 ml. of 0·0125M-phosphate buffer, pH 6·2, in N_2. 1 ml. 0·002N-$I_2 \equiv 0·615$ mg. GSH.

that in the enzyme solution alone. It will be noted that while increasing the concentration of GSSG in the solution from a value of 0·325 to 0·975 mM increased the total amount of GSH formed, it did not increase the rate of the reaction. At these levels the system must be saturated with respect to GSSG. In the experiments described later we have used GSSG at a concentration of 0·65 mM, and have therefore been reasonably certain that the rate of reduction has not been limited by the concentration of GSSG. These experiments also show that addition of GSSG considerably increases the rate of production of titratable —SH groups, and suggests that in the pea, on moistening the seed, the limiting factor in the production of sulphydryl groups is the concentration of S—S compounds and not that of the enzymic activity of the tissue in transferring hydrogen or of the substrate supplying the hydrogen.

We have assumed so far that the reaction is enzymic, and the following experiment (Fig. 2) shows that extracts which had previously been heated to 100° and held at this temperature for 2–3 min. were unable to reduce GSSG and showed no increase in titratable —SH groups over a period of 20 hr. The enzymic character of the reaction is, however, more clearly demonstrated in the next experiments.

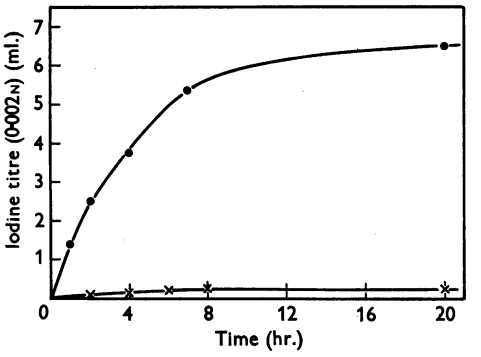

Fig. 2. Destruction of reducing activity of an undialysed pea extract by heat. ●—●, undialysed enzyme; × — ×, enzyme solution heated to 100° for 1–2 hr. GSSG in all solutions, 0·65 mM.

Reduction of glutathione in dialysed extracts of ungerminated peas

When extracts from peas were dialysed against 0·025M-phosphate buffer, pH 6·2, at + 1° for periods varying from 17 to 72 hr. the ability of the dialysed extract to reduce GSSG was gradually lost. The

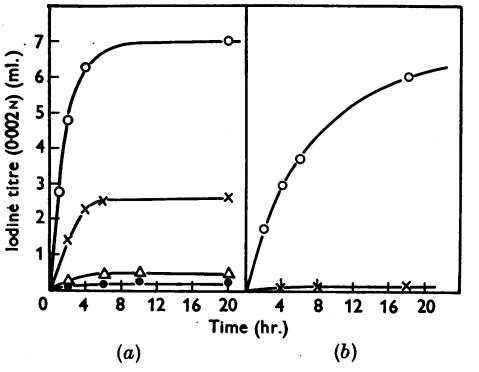

Fig. 3. (*a*) The effect of dialysis on the reduction of GSSG by an extract of dried ungerminated peas. ○—○, undialysed enzyme; ×—×, dialysed 17 hr.; △—△, dialysed 41 hr.; ●—●, dialysed 72 hr. GSSG (0·65 mM) added to each solution. (*b*) Restoration of activity to a dialysed pea extract by the addition of a boiled and filtered pea extract prepared from an acetone extracted pea powder. ×—×, extract dialysed 72 hr.; ○—○, dialysed extract + a boiled and filtered extract of pea powder. GSSG (0·65 mM) in each solution.

results of an experiment of this kind are illustrated in Fig. 3*a*. After 17 hr. dialysis, both the rate of reduction and total amount of GSSG reduced have fallen compared with the undialysed extract. After

dialysis for 41–70 hr. the enzyme extract had completely lost its power to reduce GSSG.

That this failure to reduce GSSG was not due to inactivation of an enzyme during dialysis is shown by the fact that the addition of a boiled and filtered aqueous extract from fresh acetone-extracted powder restored the ability of even the longest dialysed preparation to reduce GSSG (Fig. 3*b*).

These experiments suggested therefore that at least two factors were involved, an enzyme and a dialysable substance, presumably the substrate of the enzyme concerned. Further work was therefore carried out with the object of isolating and identifying the dialysable component.

Fractionation of acetone powder

The acetone-extracted powder prepared from the original pea meal was used as starting material. This powder was extracted with 0·0125 M-phosphate buffer (pH 6·3) for a period of 5–10 min. During this period the mixture was well stirred to facilitate extraction of water-soluble components. The powder was extracted with buffer rather than with water, for it was found that this gave a better coagulation and separation of protein material. The mixture was then centrifuged to remove starch and some insoluble protein, and the supernatant fluid was brought rapidly to the boil to inactivate enzymes and then cooled rapidly in an ice bath. This extract, when added to a dialysed enzyme preparation, restored its power of reducing GSSG.

The next stage in the fractionation consisted of adding ethanol to a concentration of 40% at room temperature. This led to a further separation of protein material without appreciably decreasing the activity of the extract. The ethanol concentration was next increased to 90% and the solution left at +1° for 4–12 hr. The whitish precipitate which separated was removed by centrifugation and redissolved in water. On testing the activity of this solution it was found to have lost about one-third of its activity. The remaining activity was found in the fraction soluble in 90% ethanol. However, despite this loss in activity, further fractionation was carried out on this fraction insoluble in 90% ethanol since considerable purification had been accomplished by this procedure.

The fraction insoluble in 90% ethanol still contained some protein material which could be precipitated by half and full saturation with $(NH_4)_2SO_4$. Tests on the protein material, however, showed it to be completely inactive. Most of the protein could be removed by adjusting the pH of the solution to 3·8, without much loss in activity in the supernatant. This solution contained some inorganic P which was removed at this stage by titration with $Ba(OH)_2$ to pH 8·3, allowing to stand at +1° for 1–2 hr. and removing the insoluble Ba salts by centrifugation. This procedure did not reduce the activity of the solution.

Further purification of the deproteinized fraction was next carried out by passing the solution through a cation-absorbing resin (Zeo-Karb 215), and the activity of the filtrate compared with that of the extract before treatment with the resin. The filtrate, when tested, showed a loss of activity, in that when added to a dialysed enzyme preparation, the rate of reduction of added GSSG was much slower, although the total amount of GSSG eventually reduced was the same (Fig. 4*a*).

This suggested the possibility that more than one component was necessary to promote full activity of the dialysed enzyme preparation, and that, besides a hydrogen donor, certain co-factors were necessary.

The fact that Zeo-Karb resin removes cations, together with the known activating effect of such ions on enzyme systems, led us to determine whether the decrease in activity of the extract, after passing through the Zeo-Karb resin, was due to this cause. The activity of this fraction was therefore re-tested with the addition of a number of inorganic salts.

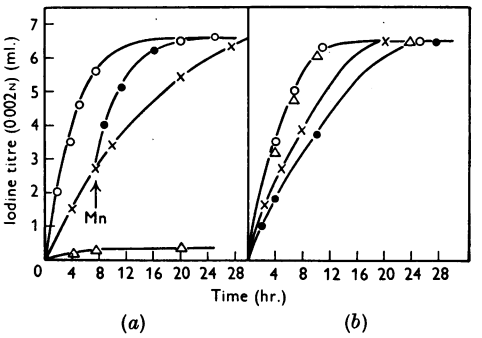

Fig. 4. (*a*) The catalytic influence of Mn on the reduction of GSSG by dialysed pea extract. (*a*) △—△, dialysed enzyme; ○—○, dialysed enzyme + substances from an aqueous extract, soluble in 40% ethanol, insoluble in 90% ethanol (90% ethanol-insoluble fraction); ×—×, dialysed enzyme + 90% ethanol-insoluble fraction after treatment with Zeo-Karb resin (Zeo-Karb fraction); ●—●, addition of Mn, $0·4 \times 10^{-3}$ M. (*b*) ●—●, dialysed enzyme + Zeo-Karb fraction; ×—×, dialysed enzyme + Zeo-Karb fraction + Mn, $0·4 \times 10^{-4}$ M; △—△, dialysed enzyme + Zeo-Karb fraction + Mn, $0·4 \times 10^{-3}$ M; ○—○, dialysed enzyme + Zeo-Karb fraction + Mn, 4×10^{-3} M. GSSG, 0·65 mM, added to all solutions.

Mn and Mg were found to be most active of the salts tried, Co and Zn salts in equivalent concentration having little catalytic effect. The results (Fig. 4) with Mn show that, under the conditions of these experiments, the minimum concentration of the metal needed to obtain the maximum effect was of the order of 0·04 mM. It seems clear, therefore, that the loss of activity of the extract on passing through the Zeo-Karb resin may have been due to the removal of metallic ions such as Mn. It will be noted that some activity with these Zeo-Karb fractions was always obtained without the addition of metallic salts, a result which may be attributed to residual traces of such metals remaining in the dialysed enzyme preparations. The difficulty of completely removing all such from enzyme preparations by prolonged dialysis is well known, and this has not yet been attempted.

Adsorption on Dowex 2 resin

The preceding experiments showed that the loss of activity on treatment with the Zeo-Karb resin was due to the removal of certain cations. The fraction was therefore further purified by passing it through a column of an anion-absorbing resin (Dowex 2). The filtrate passing this resin when tested showed no activity, indicating the absorption of the active components on the anion resin. This suggested that the active

components were probably acidic in character. However, on elution of the Dowex column with H_2SO_4, the tests on the various fractions obtained showed that the activity had been seriously reduced. This finding suggested either that the component sought had been destroyed or not eluted from the column, or that the lack of activity was due to the partial separation of two components from one another.

At this stage it seemed more profitable to examine the fractions already known to be active, to determine if it were possible to detect the presence of a hydrogen donor, which should be present in reasonable amounts, rather than attempt to separate other co-factors which might only be present in very small amounts. To do this we used the methods of paper chromatography to examine not only the fractions eluted from the Dowex column, but also the earlier fractions treated with the Zeo-Karb resin alone.

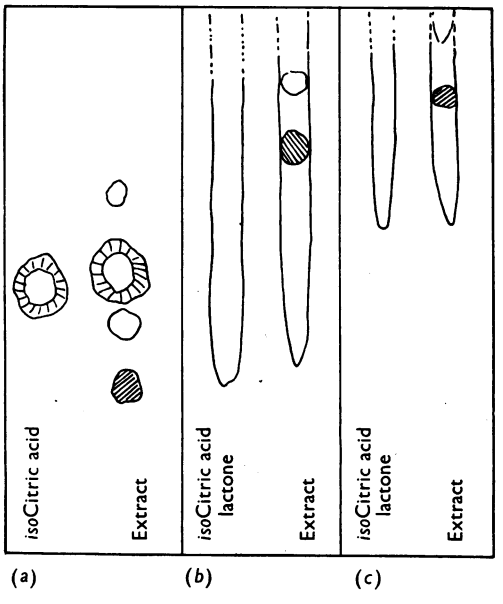

Fig. 5. Chromatograms of extracts of ungerminated pea seeds. (*a*) Chromatogram of the Zeo-Karb filtrate fraction from pea seeds. Solvent: propanol/NH_3/water (60 : 30 : 10). Spraying reagent, ammoniacal $AgNO_3$. Running time, 18 hr. *iso*Citric acid as marker. (*b*) Chromatogram of Zeo-Karb filtrate fraction after treatment with H_2SO_4 to convert *iso*citric acid to its lactone. Solvent: water-poor phase of a butanol/water/acetic acid (50 : 40 : 10) mixture. Spraying reagent, ammoniacal $AgNO_3$. Running time, 18 hr. *iso*Citric acid lactone as marker. (*c*) Chromatogram of decomposed insoluble Pb salts from Zeo-Karb filtrate fraction after treatment with H_2SO_4. Solvent and spraying reagents as in (*b*). Running time, 9 hr. *iso*Citric acid lactone as marker. Shaded areas indicate reduction of silver reagent.

Analysis by paper chromatography

With the knowledge that the unknown components in the extracts were likely to be acidic in character, we carried out an analysis to detect and identify the organic acids present. The method used was one designed by Hanes & Isherwood (unpublished) of developing a chromatogram on paper of the ammonium salts of the acids by means of a propanol/ammonia/water solvent (60 : 30 : 10). After running the acids, their position on the chromatogram can be revealed either by spraying with an alkaline solution of thymol blue or with ammoniacal silver nitrate and heating. With the first reagent the acid is indicated as a yellow spot on a blue background, and with the second either as a white or brown spot, depending on whether the acid has reducing properties.

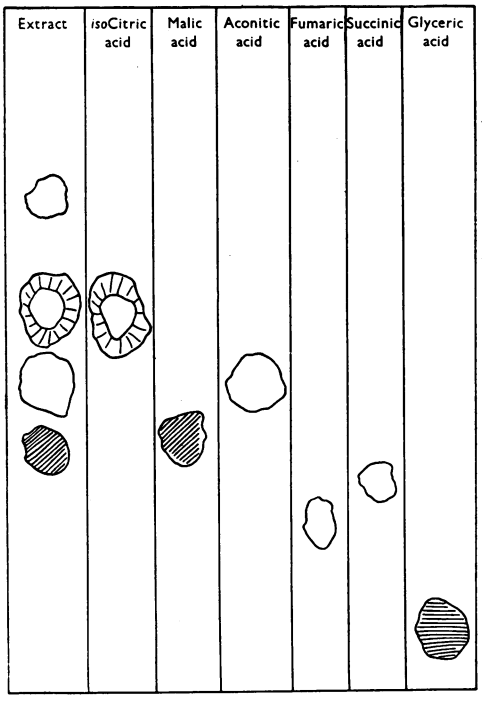

Fig. 6. Chromatogram of the decomposed insoluble Pb salts from a formic acid ethanol extract of pea seeds. Solvent: propanol/NH_3/water (60 : 30 : 10). Spraying reagent, ammoniacal $AgNO_3$. Running time, 18 hr. *iso*-Citric, malic, aconitic, fumaric, succinic and glyceric acids as markers. Shaded areas indicate reduction of silver reagent.

When the technique was applied to (1) the fraction passing the Zeo-Karb column, or to (2) the fraction eluted from the Dowex 2 resin, there was evidence of the presence of an organic acid with an R_F value suggestive of the presence of a tricarboxylic acid. This acid could also be separated as a lead salt from the extracts.

Its position on the chromatogram suggested that it was a tricarboxylic acid similar to citric or *iso*-citric acid. Further evidence suggesting it was *iso*-citric acid was obtained by treating (1) the Zeo-Karb filtrate fraction, (2) the decomposed lead salts from

this fraction, and (3) the fraction eluted from the Dowex 2 resin, with sulphuric acid and concentrating the solutions *in vacuo*. This treatment converts *iso*citric acid into its lactone. These solutions were then chromatographed on paper using as solvent the water-poor phase from a butanol/water/acetic acid (50 : 40 : 10) mixture. With this solvent the lactone of *iso*citric acid travels faster than the acid itself and leaves a characteristic trail. The following reproductions of the chromatograms obtained (Fig. 5) show in the propanol/ammonia/water solvent an acid spot on the paper similar in position to citric or *iso*citric, and after conversion to the lactone a chromatographic pattern developed in the butanol/water/acetic acid mixture similar to that given by *iso*citric acid lactone.

Chromatograms showing the presence of *iso*citric acid, together with malic and aconitic acids, were obtained by the following procedure. Ungerminated pea seeds were extracted with a solvent containing 5% (w/v) formic acid in 95% ethanol. The extract was concentrated *in vacuo* and taken up in water. After filtration neutral lead acetate was added, and the precipitated lead salts washed well with water. The lead salts were then decomposed by hydrogen sulphide and the solution neutralized with ammonia. A chromatogram of the acids was then developed using the propanol/ammonia/water solvent. A reproduction of the chromatogram is shown in Fig. 6.

Attempts to isolate these acids are being carried out and will be reported later.

Reduction of glutathione by dialysed pea enzyme with isocitrate

The foregoing data indicated the possibility that *iso*citrate was present in all of our active fractions. Experiments were therefore set up to test whether *iso*citrate could act as a hydrogen donor for the reduction of GSSG by dialysed extracts from peas. It was realized from the work of Ochoa (1945) that a coenzyme, probably Co II, as well as manganese would also be necessary. Accordingly we studied the reduction of GSSG by a dialysed enzyme preparation to which (1) *iso*citrate, (2) manganese and (3) Co I and Co II were added.

The results of an experiment of this nature are shown in Fig. 7*a*. *iso*Citrate and manganese added initially to the enzyme extract caused no reduction of GSSG. After 1·5 hr. Co I was also added without any significant result. After a further period of 2·5 hr. Co II was added. The addition of Co II caused a rapid reduction of GSSG, the reaction reaching equilibrium when most of the GSSG had been reduced. A similar experiment, illustrated in Fig. 7*b*, shows that when Co II and manganese were added initially there was no reduction of GSSG until *iso*citrate was added. It was further shown that the presence of manganese doubles the rate of the reaction. As pointed out earlier, the fact that the addition of *iso*citrate and Co II alone when added to our dialysed enzyme preparation reconstituted a system which reduced GSSG, though at a slower rate than when manganese was also added, seems to indicate that our dialysed enzyme was not completely free of metallic catalysts.

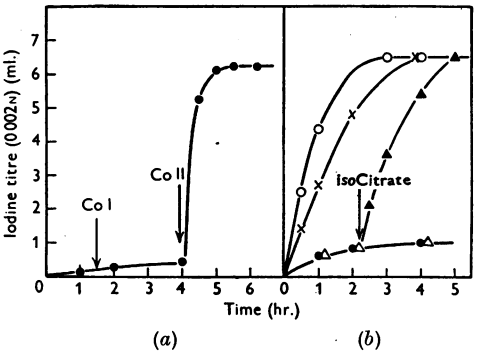

Fig. 7. The influence of *iso*citrate, Co II, Co I and Mn on the reduction of GSSG by a dialysed pea extract. (*a*) Enzyme solution contained initially D-*iso*citrate (0·005 M), Mn, $1·6 \times 10^{-3}$ M. After 15 hr. Co I (10 μg./ml.), and after a further 25 hr. Co II (5 μg./ml.) were added. (*b*) ●—●, dialysed enzyme + Co II (5 μg./ml.); △—△, dialysed enzyme + Co II + Mn ($1·6 \times 10^{-3}$ M); ▲—▲, after 2·25 hr. D-*iso*citrate added (0·005 M); ○—○, dialysed enzyme + Co II, D-*iso*citrate + Mn; ×—×, dialysed enzyme + Co II, D-*iso*citrate. GSSG, 0·65 mM, present in all solutions.

The most obvious interpretation of these facts is that the reduction of GSSG is dependent on the production of reduced Co II, this being produced by reactions similar to those described by Ochoa (1945). We may thus tentatively write the reactions involved as

(1) *iso*citrate + Co II ⇌ oxalosuccinate + reduced Co II,

$$\text{(2) oxalosuccinate} \overset{\text{Mn}}{\rightleftharpoons} \alpha\text{-ketoglutarate} + CO_2,$$

(3) reduced Co II + GSSG → Co II + 2GSH.

Table 1. *Formation of α-ketoglutarate during the reduction of glutathione*

(*System.* Dialysed enzyme + Co II (5 μg./ml.) + D-*iso*citrate, 1·25 mM + Mn, 1·6 mM + GSSG, 0·65 mM. A control solution containing all constituents with the exception of GSSG was also set up, and the small amounts of α-keto acid formed in this solution subtracted from that of the experimental solution.)

Time (hr.)	GSSG reduced (% of total added)	α-Ketoglutaric acid formed (% of theory)
0·25	12	13
1·25	52	56
3	95	106
4	97	110

Reaction (1) is catalysed by *iso*citric dehydrogenase, and reaction (2) by oxalosuccinic decarboxylase with manganese as co-factor.

Using the method of Friedemann & Haugen (1943), we have been able to show that an α-keto acid is formed during these reactions. On the assumption that the acid is α-ketoglutaric acid we have further shown that it is formed in the amounts expected on the basis of these reactions (Table 1).

Preliminary studies of the reaction between reduced Co II and GSSG are given in a later section of this paper.

Reduction of glutathione by dialysed pea enzyme with malate

The previous experiments had shown the presence in our enzyme preparations of an *iso*citric dehydrogenase, reducing Co II but not Co I. We suspected that other dehydrogenases capable of reducing Co II would, if present in our enzyme preparation, likewise catalyse the reduction of GSSG. Vennesland and her co-workers (Vennesland, Gollub & Speck, 1949; Vennesland, 1949; Conn, Vennesland & Kraemer, 1949) have shown the presence of a dehydrogenase catalysing the oxidation of malate to pyruvate in wheat germ, pea seeds, spinach and in several root vegetables. Co II is necessary and the reactions involved have been shown to be

$$\text{malate} + \text{Co II} \rightleftharpoons \text{oxaloacetate} + \text{reduced Co II},$$

$$\text{oxaloacetate} \overset{\text{Mn}}{\rightleftharpoons} \text{pyruvate} + CO_2.$$

The malate dehydrogenase was found to be Co II specific. These series of reactions are similar to those in which *iso*citrate is the hydrogen donor.

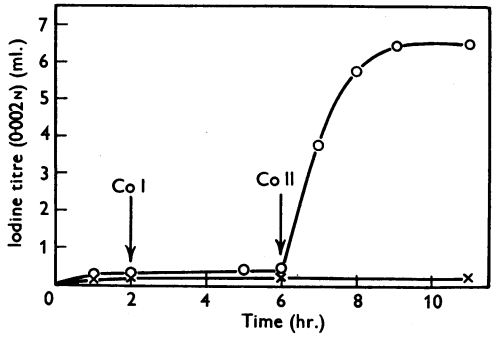

Fig. 8. The influence of Co I and Co II on the reduction of GSSG by malate. ×—×, dialysed enzyme; ○—○, dialysed enzyme + DL-malate (0·008 M) + Mn ($1·6 \times 10^{-3}$ M). After 2 hr. Co I (10 μg./ml.) added, followed by addition of Co II (5 μg./ml.) after 6 hr. GSSG, 0·65 mM, in all solutions.

Experiments were therefore set up, similar to those we have reported, using malate instead of *iso*citrate. The results illustrated in Fig. 8 show that in the presence of malate and manganese with the dialysed enzyme there is no reduction of GSSG until Co II is added. The data also show that Co I cannot serve as a coenzyme in the system.

Further experiments (Fig. 9) show (1) that Co II alone or with manganese is ineffective, in the absence of malate, and (2) that manganese acts as a co-factor. The effect of manganese in increasing the rate of the reduction was even greater than with the corresponding experiments with *iso*citrate.

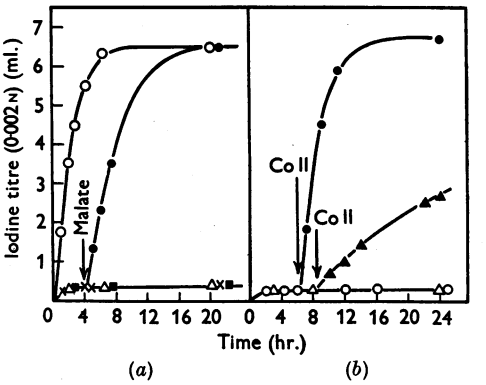

Fig. 9. Reduction of GSSG by dialysed pea extract with additions of malate, Mn, Co I and Co II. (*a*) ×—×, dialysed enzyme; △—△, dialysed enzyme + Mn ($1·6 \times 10^{-3}$ M); ■—■, dialysed enzyme + Co II (10 μg./ml.) + Mn; ●—●, after 4 hr. DL-malate (0·008 M) added; ○—○, dialysed enzyme + Co II + Mn + malate. (*b*) △—△, dialysed enzyme + DL-malate (0·008 M); ▲—▲, after 8 hr. Co II (10 μg./ml.) added; ○—○, dialysed enzyme + malate + Mn ($1·6 \times 10^{-3}$); ●—●, after 6 hr. Co II (10 μg./ml.) added. GSSG, 0·65 mM, in all solutions.

We have shown that pyruvic acid is formed during the reduction of GSSG to GSH by this enzyme system. In experiments similar to those above both GSH and pyruvic acid were estimated, the latter by the method of Friedemann & Haugen (1943). The amounts of pyruvic acid formed (Table 2) agreed well with those expected on the basis of the overall reaction

$$\text{Malate} + \text{GSSG} \overset{\text{Co II} + \text{Mn}}{\rightleftharpoons} 2\text{GSH} + \text{pyruvate} + CO_2.$$

Table 2. *Formation of pyruvate during the reduction of glutathione*

(*System.* Dialysed enzyme + Co II (5 μg./ml.) + DL-malate, 2 mM + Mn, 1·6 mM + GSSG, 0·65 mM. A control solution containing all constituents with the exception of GSSG was also set up, and the small amount of pyruvic acid formed in the solution subtracted from the total pyruvic acid formed in the experimental solution.)

Time (hr.)	GSSG reduced (% of total added)	Pyruvic acid formed (% of theory)
1·5	46	42
2·23	65	59
3·5	73	72
5·0	92	87

The factor common to these experiments and those described for *iso*citrate is the formation of reduced Co II. It seems clear, therefore, that the essential reaction in the reduction of GSSG is

$$CoH_2II + GSSG \rightleftharpoons Co II + 2GSH.$$

Oxidation of reduced coenzyme II by glutathione

This reaction was studied by measuring the changes in absorption at 340 mμ. of reduced Co II, prepared enzymically as described in the first section of this paper, using a Hilger spectrophotometer. When GSSG was added to reduced Co II in 0·1 M-phosphate, pH 6·5, there was no oxidation of the coenzyme, and no production of GSH. When, however, the pea enzyme was added there was a rapid oxidation of reduced Co II, as determined by the decrease in absorption at 340 mμ. (Fig. 10*b*). In a similar experiment in which the pea enzyme was added to reduced Co II, there was little, if any, oxidation until GSSG was added, when a rapid oxidation of the reduced enzyme resulted (Fig. 10*a*). These experiments show clearly that the reaction reduced Co II + GSSG $\rightleftharpoons$ 2GSH + Co II is catalysed by an enzyme present in ungerminated pea seeds.

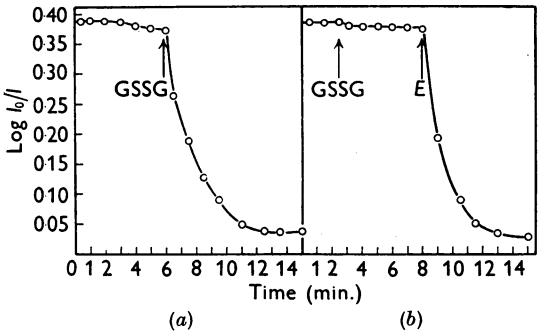

Fig. 10. The oxidation of reduced Co II by GSSG. (*a*) The system contained initially 1·5 ml. 0·1 M-phosphate buffer, pH 6·50, 0·5 ml. of dialysed pea enzyme, 130 μg. reduced Co II in a total of 3 ml. After 6 min. 250 μg. of GSSG were added. (*b*) The system contained initially 1·5 ml. 0·1 M-phosphate buffer, and 130 μg. reduced Co II in a total of 3 ml. After 3 min. 250 μg. GSSG, and after a further 5 min. the enzyme extract (*E*) were added. The reaction was followed by measuring changes in absorption at 340 mμ. in a Hilger spectrophotometer.

In further experiments it was shown that the amount of reduced Co II oxidized agreed with the equation above. Thus, in an experiment in which 0·115 μmol. of GSSG was added to a solution containing 0·17 μmol. of reduced Co II, 0·11 μmol. of Co II was produced.

We have also studied the reverse reaction, but have not been able to detect any reduction of Co II when six times the equivalent amount of GSH is added to Co II in the presence of the pea enzyme. This indicates that the equilibrium point of the reaction is very much in favour of the formation of GSH. A more detailed study of the characteristics of this enzyme is now being made, and these results will be reported later.

Experiments with coenzyme I

Bukin (1943) claimed that reduced Co I would reduce GSSG by a non-enzymic reaction, but we have not been able to confirm this. No changes in the absorption at 340 mμ. in a solution containing reduced Co I and GSSG were observed using a Hilger spectrophotometer. The Co I was reduced chemically with sodium dithionite. Nor in similar experiments could we detect any formation of reduced Co II when GSH was added to Co I.

Adler & Sreenivasaya (1937) found that pea seeds contained a formic dehydrogenase which was Co I specific. We have confirmed this, and in addition have found that the dialysed enzyme preparation contained an even more active ethanol dehydrogenase. Both enzymes reduced Co I as shown by the increase in absorption at 340 mμ. after addition of either formate or ethanol to the enzyme solution to which Co I had been added. The activities of these enzymes were measured by the Thunberg methylene-blue technique; the results of such an experiment are shown in Table 3. There was a rapid reduction of methylene blue in the presence of formate or ethanol provided Co I was also added. There was no reduction in the absence of the enzyme.

Table 3. *Reduction of methylene blue*

(Each tube contained 0·5 ml. dialysed enzyme, 1·5 ml. 0·025 M-phosphate buffer, pH 6·3, +0·5 ml. 1/10,000 methylene blue. The final concentration of coenzyme I was 100 μg./ml., of ethanol 9 mM and of sodium formate 2 mM. The final volume was 3 ml. in each tube.)

Dialysed enzyme	Sodium formate	Ethanol	Co I	Reduction time (min.)
+	+	−	+	10
+	+	−	−	>120
+	−	−	+	120
+	−	+	+	4
+	−	+	−	>120

It seems clear that the enzyme extract contained both active formic and ethanol dehydrogenases reacting with Co I. Nevertheless, when ethanol or formate was added to the enzyme preparation, together with Co I and GSSG, there was no reduction of GSSG. These experiments show, therefore, that dehydrogenases capable of reducing Co I cannot transfer hydrogen via Co I to GSSG, presumably due to lack of a suitable catalyst for the reaction between reduced Co I and GSSG.

The reduction of dehydroascorbic acid by the glutathione iso*citrate system*

We have shown that GSSG may be reduced by either the *iso*citrate or malate dehydrogenase systems present in ungerminated peas, and we felt it to be of interest to try to show the transfer of hydrogen via such systems to dehydroascorbic acid.

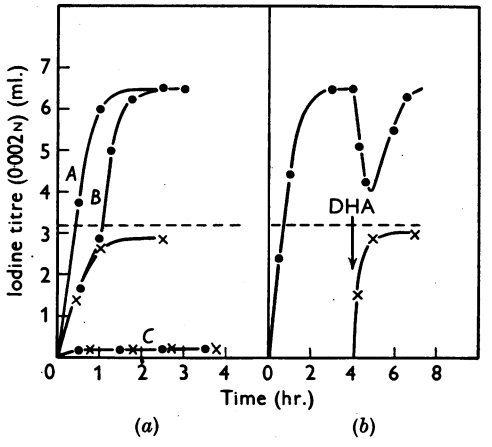

Fig. 11. The reduction of dehydroascorbic acid by dialysed pea extract. (*a*) *A*, dialysed enzyme + D-*iso*citrate (0·005 M). Mn ($1{\cdot}6 \times 10^{-3}$ M) + Co II ($10\,\mu$g./ml.) and GSSG (0·65 mM). *B*, the same as *A* + dehydroascorbic acid. *C*, Dialysed enzyme + *iso*citrate + Mn + Co II + dehydroascorbic acid (0·1 mg./ml.). ●—●, GSH; ×—×, ascorbic acid. (*b*) Dialysed enzyme + *iso*citrate + Co II + Mn + GSSG. After 4 hr. dehydroascorbic acid (0·1 mg./ml.) added. In both graphs the dotted line indicates the amount of dehydroascorbic acid added. ●—●, GSH; ×—×, ascorbic acid.

A dialysed enzyme preparation was set up with *iso*citrate, Co II, manganese and GSSG, and the reduction of GSSG followed as in previous experiments. To one tube no further addition was made; to the other a known amount of dehydroascorbic acid. Estimations of GSH in the first case and of GSH and ascorbic acid in the second case were carried out. The results (Fig. 11*a*) show that, in the absence of dehydroascorbic acid, a rapid formation of GSH occurs. In the presence of dehydroascorbic acid the accumulation of GSH occurs more slowly. When the reduction of dehydroascorbic acid is complete, the rate of accumulation of GSH increases and continues until it reaches the same value as that reached in the control solution. In the absence of GSSG there was no reduction of dehydroascorbic acid. These observations are the result of two opposing reactions: the reduction of GSSG to GSH, which proceeds at the same rate in the solution to which dehydroascorbic acid is added as in the control, and the oxidation of GSH by dehydroascorbic acid, which reduces the net accumulation of GSH. This is borne out by the curves (Fig. 11*a*) where, in the initial stages of the reaction, the sum of GSH and ascorbic acid produced is very nearly equal to the amount of GSH produced in the control experiment.

In a following experiment (Fig. 11*b*) the reduction of dehydroascorbic acid at the expense of GSH is again clearly shown. In this experiment the dialysed enzyme with Co II, *iso*citrate, manganese and GSSG was allowed to reach equilibrium. At this stage dehydroascorbic acid was added. There was a rapid reduction of the dehydroascorbic acid which was correlated with a fall in the GSH content. After the reduction of the dehydroascorbic acid, there was again a rise in GSH until the original value was reached.

These experiments demonstrate the transfer of hydrogen from *iso*citrate to dehydroascorbic acid in accordance with the following scheme:

$$\textit{iso}\text{citrate} + \text{GSSG} \xrightarrow{\text{Co II} + \text{Mn}} 2\text{GSH} + \alpha\text{-ketoglutarate} + \text{CO}_2$$

$$2\text{GSH} + \text{DHA} \rightarrow \text{GSSG} + \text{AA}.$$

This system, in combination with ascorbic oxidase or similar enzymes, will therefore effectively transfer hydrogen from substances such as *iso*citrate or malate, and since both are intermediates in the Krebs cycle, we have the possibility of a link here with the normal respiratory mechanisms.

DISCUSSION

Mann (1932) observed the reduction of GSSG by glucose and glucose dehydrogenase prepared from liver. He found, however, that the rate of this reaction was slow, but could be accelerated by an activator extracted from liver tissue. The nature of this activator was not disclosed.

Meldrum & Tarr (1935) observed that GSSG was reduced by enzyme extracts from blood or yeast in the presence of hexosemonophosphate and extracts containing Co II.

The work presented in this paper has shown a hydrogen transfer mechanism, whereby hydrogen from certain intermediates, derived ultimately from carbohydrate, may pass to molecular oxygen via Co II, GSSG and dehydroascorbic acid. In this respect the system provides an alternative respiratory pathway to that of cytochrome and links both GSH and ascorbic acid with Co II, which itself is one central point in the hydrogen-activating mechanisms of the cell.

We have at present no direct information how important such a system is in the general respiratory activity of the pea seed, or how active it is, in relation to that of cytochrome. As far as our knowledge goes, this enzyme system, like that of cytochrome, would be cyanide sensitive, for all the terminal

oxidases, i.e. ascorbic oxidase, polyphenolase, and peroxidase known to oxidize AA in plant tissues, are poisoned by cyanide.

The evidence that a close correlation exists between GSH and AA in plant tissues has already been summarized in the introduction. Direct evidence that there is a connexion between the state of oxidation of GSH and that of AA has recently been shown by Barker & Mapson (1951), who found that when potato tubers are subjected to an atmosphere of pure oxygen there is first a fall in the GSH content of the tissue, followed closely by a fall in AA and a rise in DHA. This evidence, although incomplete, suggests that the AA content of the tissue is dependent on the maintenance of its GSH content.

We have no indication yet of the nature of the enzyme catalysing the reaction between reduced Co II and GSSG, which by analogy with cytochrome reductase we might term glutathione reductase. From our knowledge of the nature of diaphorase and cytochrome reductase, all we may do at this stage is to speculate whether this enzyme too may be a flavoprotein.

SUMMARY

1. Extracts from ungerminated pea seeds under anaerobic conditions rapidly reduce glutathione (GSSG) to reduced glutathione (GSH). When these extracts are dialysed there is a progressive fall in the activity of the extract.

2. There is chromatographic evidence that both *iso*citric and malic acids are present in pea seeds.

3. The activity of these dialysed extracts may be restored by the addition of *iso*citrate or malate, coenzyme II (Co II) and manganese. With *iso*citrate, α-ketoglutaric acid and with malate, pyruvic acid is formed in amounts equivalent to the glutathione reduced. Coenzyme II cannot be replaced by coenzyme I.

4. Reduced coenzyme II does not reduce glutathione in solution in the absence of enzymic catalysts. Dialysed pea extracts contain an enzyme which actively catalyses the reaction

$$\text{Reduced Co II} + \text{GSSG} \rightleftharpoons \text{Co II} + 2\text{GSH}.$$

The reduction of glutathione is equivalent to the amount of coenzyme II oxidized.

5. The reverse reaction between coenzyme II and reduced glutathione could not be demonstrated, indicating that the equilibrium is in favour of the formation of reduced glutathione.

6. The presence in the dialysed pea extracts of ethanol and formic dehydrogenase enzymes, both coenzyme I specific, has been shown. These enzymes in the presence of their substrates and coenzyme I will not reduce glutathione; reduced coenzyme I will not reduce glutathione, nor was any evidence obtained of the presence of an enzyme which would catalyse the reaction.

7. Dehydroascorbic acid is rapidly reduced to ascorbic acid when it is added to a dialysed enzyme extract containing isocitrate, coenzyme II, and manganese glutathione. The reduction of dehydroascorbic acid is dependent on the production of reduced glutathione.

8. When dehydroascorbic acid reductase is present, a system is complete by which hydrogen may be transferred in turn from the hydrogen of substrates of enzymes reducing coenzyme II to glutathione and dehydroascorbic acid.

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