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The Oxidation of Glutathione by a Lipoxidase Enzyme from Pea Seeds

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Snow & Zilva (1938) were among the first to show the enzymic oxidation of glutathione (GSH) by both dialysed and undialysed cucumber juice. They also showed an appreciable oxygen uptake on adding GSH to these juices, and supplied evidence that the enzyme catalysing this reaction was different from ascorbic acid oxidase. Ames & Elvehjem (1945) showed the existence of an enzyme system oxidizing GSH in mouse-kidney homogenates, and found that this system was more active when cytochrome *c* was added. They suggested a respiratory system in which GSH and cytochrome *c* were involved. Many investigators have studied the indirect oxidation of GSH through the ascorbic acid–dehydroascorbic acid system (Szent-Gyorgyi, 1928; Hopkins & Morgan, 1936; Crook, 1941). This oxidation was found to be catalysed by the enzyme dehydroascorbic acid reductase (Crook & Hopkins, 1938; Crook, 1941).

During the course of work on the role of GSH in plant respiration, it was observed that GSH, under certain circumstances, was rapidly oxidized by dialysed extracts prepared from ungerminated pea seeds. Since the dialysed extract was devoid of ascorbic acid (AA) or dehydroascorbic acid (DHA) the oxidation could not be attributed to the action of DHA reductase. Furthermore, it was found that one of the enzyme systems concerned was cyanide-insensitive, which excludes the possibility that ascorbic acid oxidase, polyphenol oxidase or the cytochrome system could be involved.

In the present work an attempt has been made to elucidate the oxidizing systems concerned.

MATERIALS AND METHODS

Source of enzyme

Extracts were prepared from powdered dry ungerminated pea seeds, variety Laxton Superb, which had been rapidly extracted with cold acetone (Mapson & Goddard, 1951). The acetone-powder preparation was preferred to unextracted powder owing to the greater ease of subsequent extraction. Control experiments have shown that results essentially similar to those reported below were obtained from unextracted powdered pea seeds.

Dialysed enzyme

Acetone powder (30 g.) was mixed with 100 ml. of 0·01 M potassium phosphate buffer, pH 6·8, and allowed to stand 15 min. at room temperature. The suspension was centrifuged and the supernatant was dialysed against the same phosphate buffer for 48 hr. This operation was carried out at +1° and air was bubbled through the external solution to facilitate dialysis.

Determination of lipoxidase activity

The lipoxidase activity was determined by measuring the O_2 uptake in the presence of either methyl linoleate or methyl linolenate, using Warburg manometers at 25°. The volume of the reaction mixture was 3 ml.; 0·2 ml. of 20% KOH in the central well was used to absorb CO_2.

Measurement of the enzymic oxidation of GSH

This was carried out in flasks fitted with delivery tubes for bubbling air or nitrogen, shaken at a speed of about 80 cyc./min. The reaction mixture, unless otherwise stated, consisted of 15 ml. of the dialysed pea extract and 15 ml. of 0·2 M phosphate buffer, pH 6·8. At this pH the rate of the non-enzymic oxidation of GSH was low. GSH was added at

the start of each experiment, in a concentration of 0·1–0·25 mg./ml. A portion of the mixture (5 ml.) was removed at appropriate intervals and pipetted directly into 5 ml. of 4% (w/v) metaphosphoric acid and the solution centrifuged to remove the precipitated protein. The clear supernatant was then taken for the estimation of GSH. All experiments were carried out at 25°.

Determination of GSH

This was determined by the iodometric method of Fujita & Numata (1938).

Fatty acid esters

The methyl esters of linoleic, linolenic and oleic acids were obtained from the Hormel Institute, Minnesota, U.S.A.

RESULTS

Oxidation and reduction of glutathione by pea extract

In preliminary experiments, it was observed that both GSH and cysteine rapidly disappeared when they were added to a dialysed extract prepared from ungerminated pea seeds under aerobic conditions. In these experiments a few drops of octan-2-ol were added to prevent frothing. Under anaerobic conditions the disappearance of these sulphydryl compounds could not be observed. The disappearance of the reduced form appeared to be due to the oxidation to the disulphide form, for GSH could be regenerated under anaerobic conditions on the addition of malate, TPN and manganese, thus constituting the reducing system, malic dehydrogenase–glutathione reductase, previously described by Mapson & Goddard (1951). These reactions are illustrated in Fig. 1*a*.

The oxidation of both GSH and cysteine was found to be enzymic in character for, in addition to other properties described later, the rate of oxidation in the presence of octan-2-ol and a dialysed extract which had been previously heated at 100° for 2 min. was no greater than the rate of oxidation in buffer solution alone.

It was noteworthy that octan-2-ol appeared to be necessary to initiate this reaction. Experiments showing this are illustrated in Fig. 1*b*. It will be seen that the rate of oxidation of GSH added to the dialysed pea extract was the same as the rate of the reaction in buffer alone. When octan-2-ol was added 30 min. after beginning the experiment, the rate of oxidation was increased tenfold.

Oxygen uptake of the enzyme system involved

A study of the oxygen uptake of the system has thrown further light on the reactions involved. The ability of these extracts to catalyse the oxidation of GSH appeared to run parallel to their ability to consume oxygen. The O_2 taken up by the dialysed pea extract with or without the addition of GSH was very small, but was greatly augmented on addition of octan-2-ol. The enzymic nature of the reaction was again indicated by the complete destruction of the ability of the extract to take up

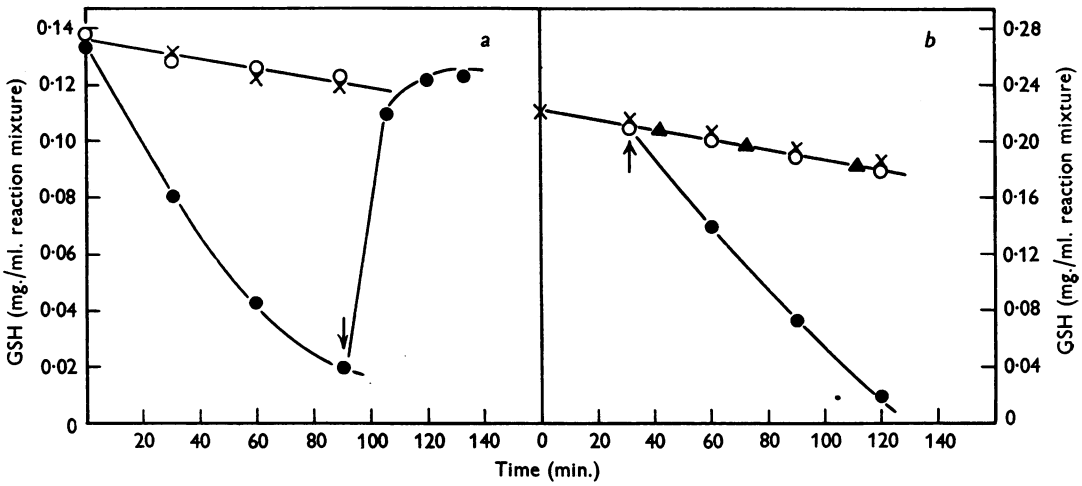

Fig. 1. (*a*) Oxidation and reduction of GSH by pea extract. ●, Green dialysed extract + octan-2-ol (0·01 ml./ml.) + GSH (0·14 mg./ml.); ↓, after 90 min., malate, TPN and $MnCl_2$ were added; ○, boiled green dialysed extract + octan-2-ol + GSH; ×, buffer solution + octan-2-ol + GSH. (*b*) Effect of octan-2-ol on the oxidation of GSH by a dialysed pea extract. ○, Extract + GSH (0·23 mg./ml.); ●, after 30 min. octan-2-ol (0·01 ml./ml.) was added; ×, boiled extract + GSH; ▲, after 30 min. octan-2-ol was added.

O_2 after heating at 100°. One further fact emerged from these studies, namely that the addition of GSH to these extracts in varying amounts did not increase either the rate or the total amount of O_2 consumed (Fig. 2). This indicated that (1) GSH was not being oxidized as a result of direct reaction with molecular O_2, and that (2) there was another substrate present in the dialysed pea extract which was being oxidized in the presence of an enzyme only when octan-2-ol was added. The most likely explanation of these results was that GSH was not being directly oxidized, but was being oxidized in a secondary reaction at the expense of some other oxidant produced in the extract, and that this oxidant was not regenerated in a form which could again be oxidized. This oxidant must have been formed in the dialysed extract under the influence of octan-2-ol. It seemed unlikely that octan-2-ol was itself responsible, in view of the fact that inert solvents like benzene, although less effective, acted similarly. Attempts were therefore made in the following experiments to ascertain the nature of both enzyme and substrate.

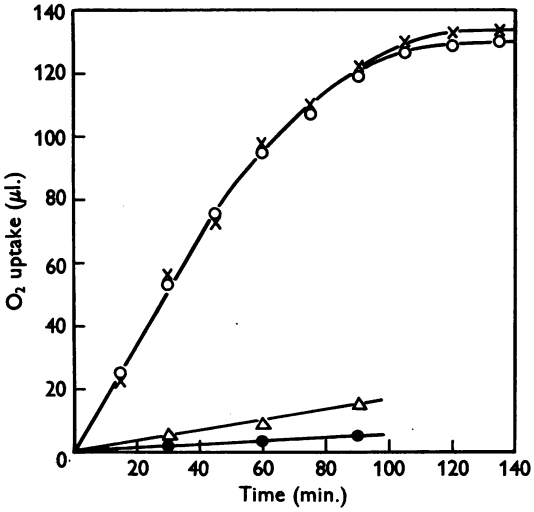

Fig. 2. Effect of octan-2-ol and GSH on the O_2 consumption by a dialysed pea extract. ●, Extract alone; △, extract + GSH (0·3 mg./ml.); ○, extract + octan-2-ol (0·01 ml./ml.); ×, extract + octan-2-ol + GSH. Each Warburg flask contained 1·5 ml. extract and 0·2 ml. 20% KOH (in central well) in a total volume of 3 ml.

Separation of enzyme and substrate

The separation of two factors, an enzyme and oxidizable substrate in the pea extract, was achieved as follows. An acetone powder from peas (30 g.) was extracted twice with 50 ml. benzene after the addition of 10 ml. water. The green benzene extract was concentrated under reduced pressure at 45°. The colourless residue of extracted powder was extracted at room temperature with 100 ml. 0·01 M phosphate buffer, pH 6·8, centrifuged and the supernatant dialysed against 5 l. of the same buffer for 48 hr. at 1°. This preparation is referred to as the 'colourless dialysed extract'.

The colourless dialysed extract did not induce the oxidation of GSH, nor was any O_2 uptake observed

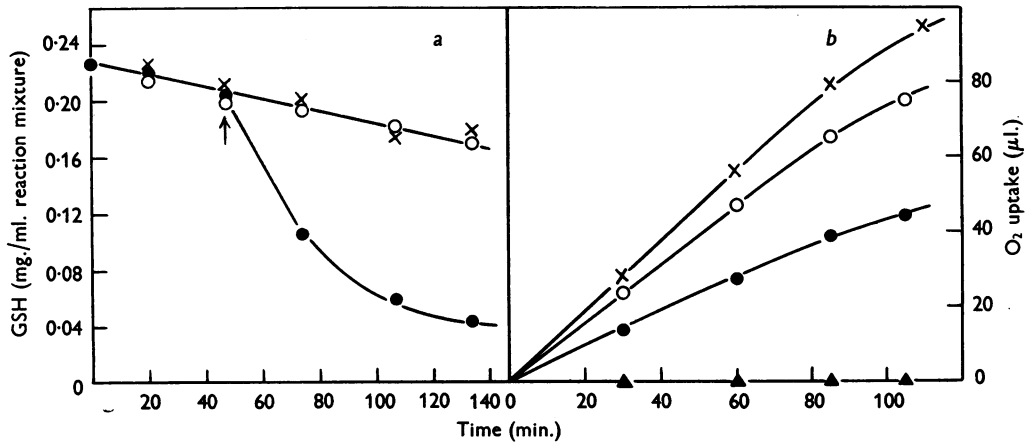

Fig. 3. (*a*) Oxidation of GSH by a defatted pea extract. ○, Colourless dialysed extract + octan-2-ol; ●, colourless dialysed extract; ↑, after 45 min. green benzene extract was added; ×, boiled colourless dialysed extract + octan-2-ol + green benzene extract. (*b*) Effect of octan-2-ol and the green benzene extract on the O_2 consumption of a defatted pea extract. ▲, Colourless dialysed extract + octan-2-ol; ●, colourless dialysed extract + 0·02 ml. benzene extract; ○, colourless dialysed extract + 0·04 ml. benzene extract; ×, colourless dialysed extract + 0·06 ml. benzene extract. Each Warburg flask contained 1·5 ml. extract and 0·2 ml. 20% KOH (in central well) in a total volume of 3 ml.

either in the presence or absence of octan-2-ol or benzene. On the other hand, when an amount of the green benzene extract, equivalent to that present in the original material, was added, the power to catalyse both the oxidation of GSH and to consume O_2 was restored (Fig. 3). As the benzene vapour interfered in the manometric experiments, the benzene was evaporated *in vacuo* and the residue was taken up in octan-2-ol.

Since the activity of the colourless dialysed extract was found to be thermolabile while that of the green benzene extract was thermostable, it appeared probable that the former fraction contained the enzyme and the latter the substrate.

Nature of the substrate

The green benzene extract contained appreciable amounts of chlorophyll and carotenoids, the colours of which were bleached during the oxidation of GSH. It was at first considered that these compounds might have been responsible for some of the effects observed. This, however, was shown not to be the case, for neither chlorophyll prepared from nettle leaves according to the method of Willstätter & Stoll (1928), nor carotenoids extracted from carrots, had any effect on either the O_2 uptake or the oxidation of GSH when added to the colourless dialysed enzyme extract.

Saponification of the benzene extract indicated that the substrate was associated with the fatty acid fraction. The green benzene extract was treated with ethanolic KOH at room temperature in the complete absence of oxygen. After separating the benzene layer containing the unsaponifiable matter, the aqueous phase was acidified with hydrochloric acid and the fatty acids were extracted with light petroleum (b.p. 40–60°). The light petroleum was removed *in vacuo*, and each of the two fractions so obtained, i.e. the unsaponifiable and the fatty acid fraction was added separately to the dialysed colourless extract, and observations were made of the effect of these additions on both the O_2 uptake and the rate of oxidation of GSH. The results showed, however, that the fatty acid fraction alone stimulated the O_2 consumption and catalysed the oxidation of GSH.

These observations focused our attention on the possibility that the oxidizing system was another example of lipoxidase activity. Pea seeds have been reported to contain an enzyme, lipoxidase, capable of oxidizing certain unsaturated fatty acids containing interrupted double bonds forming hydroperoxides of the type

$$(—CH_2.\underset{\displaystyle OOH}{\underset{|}{CH}}.CH=CH.CH=CH.CH_2—)$$

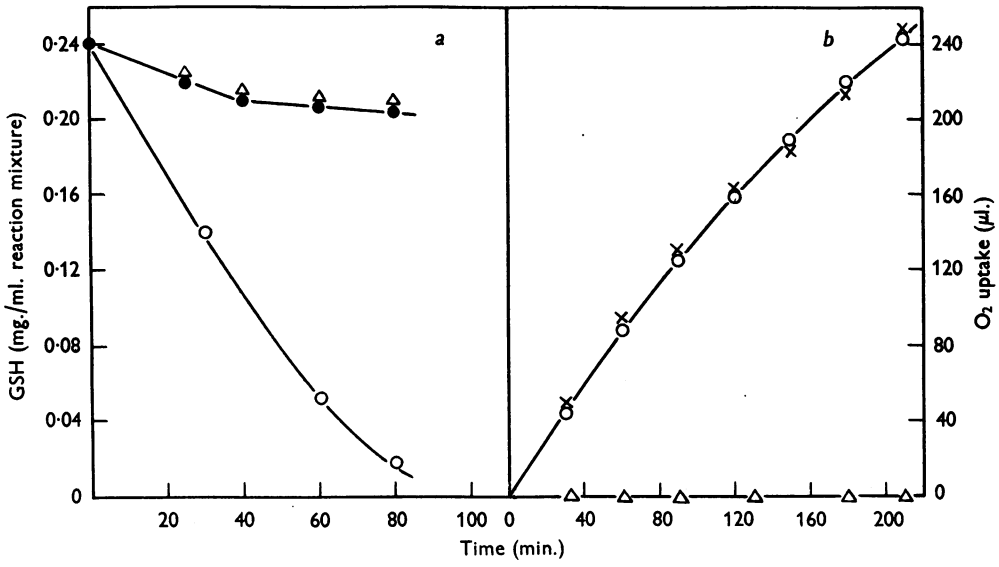

Fig. 4. (*a*) Effect of methyl linoleate on the oxidation of GSH by a defatted pea extract. ○, Colourless dialysed extract + methyl linoleate (1 mg./ml.) + GSH (0·24 mg./ml.); △, boiled colourless dialysed extract + methyl linoleate + GSH; ●, colourless dialysed extract + methyl oleate + GSH. (*b*) Effect of methyl linoleate on the O_2 uptake by a defatted pea extract. ○, Colourless dialysed extract + methyl linoleate (2 mg./flask); ×, colourless dialysed extract + methyl linoleate + GSH; △, boiled colourless dialysed extract + methyl linoleate.

(Reiser & Fraps, 1943; Sumner, 1942). It was also shown that ascorbic acid, chlorophyll, and carotenoids could undergo coupled oxidation when they were added to the lipoxidase system. Evidence that the reaction under investigation was a coupled oxidation involving the lipoxidase system was obtained when it was found that adding methyl linoleate or linolenate to either the green dialysed extract or the defatted colourless dialysed extract had the same effect as adding octan-2-ol to the green dialysed extract in stimulating both the oxidation of GSH and uptake of O_2 (Fig. 4). Moreover, the relation between concentration of fatty acid added and the rate of oxidation of GSH was that which would be expected on the supposition that a lipoxidase enzyme was involved, from which data the substrate concentration at half maximal rate (K_m) was found to be 5×10^{-4} M.

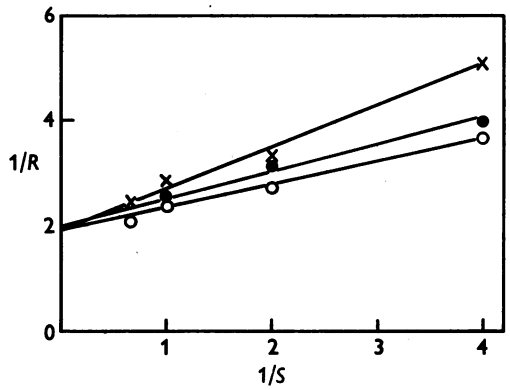

Fig. 5. Competitive inhibition of GSH oxidation in pea extract by methyl oleate. ○, No methyl oleate; ●, 1 mg./ml. methyl oleate; ×, 2 mg./ml. methyl oleate. S = concentration of methyl linoleate (mg./ml.); R = rate of reaction (mg. GSH oxidized/ml./hr.).

One of the most characteristic properties of the lipoxidase enzyme is its inability to oxidize oleic acid (Holman & Bergström, 1951) and the corresponding ability of oleic acid to act as a competitive inhibitor of the oxidation of fatty acids containing interrupted double bonds (Holman, 1947). The effect of oleic acid on the oxidation of GSH in the presence of linoleic acid and a dialysed pea extract was therefore investigated. Oleic acid not only failed to promote the oxidation of GSH by pea extracts, but acted as an inhibitor. The nature of this inhibition was shown by determining the rate of oxidation of GSH using different concentrations of methyl linoleate in the presence of different concentrations of methyl oleate. Expressing the results according to the method of Lineweaver & Burk (1934), i.e. by plotting the reciprocal of the concentration of substrate (methyl linoleate), $1/S$, against the reciprocal of the rate of oxidation of GSH, $(1/R)$, in the presence of different concentrations of inhibitor (methyl oleate), straight lines with the same intercept on the ordinate were obtained (Fig. 5), but with slope increasing with increasing oleate concentration. This shows that the inhibition by oleate was competitive and confirms the view that the reaction under investigation was due to the activity of the lipoxidase enzyme.

It was also found that the colourless enzyme extract with the addition of either linoleic or linolenic acid catalysed the oxidation of ascorbic acid, chlorophyll and carotenoids. These facts are in agreement with previously reported work on the properties of lipoxidase (Holman, 1947; Bergström & Holman, 1948; Strain, 1941).

The conclusion that the oxidation of GSH, in the system under investigation, was due primarily to lipoxidase was supported by further experiments which showed that the characteristics of the enzyme were very similar to those already reported for lipoxidase.

Effect of other inhibitors

The oxidation of GSH or O_2 uptake, as catalysed by the colourless dialysed extract with methyl linoleate as substrate, was insensitive to cyanide at concentrations up to 0·01 M, and to azide, iodoacetate and 8-hydroxyquinoline in concentrations of 0·001 M. These results are in agreement with those already reported for lipoxidase.

Effect of pH

Confirmation that the enzyme responsible for both the O_2 consumption and oxidation of GSH was a lipoxidase was obtained in a study of the influence of pH. The identical effect of pH on (1) the O_2 consumption of the green dialysed extract activated by octan-2-ol, (2) the oxidation of GSH by the same mixture, and (3) the O_2 uptake of the colourless dialysed extract with methyl linoleate (lipoxidase activity), support the viewpoint that the first two reactions are also due to lipoxidase activity (Fig. 6). The optimum pH of 6·7 found here was the same as that found by Smith & Sumner (1948) for the optimum pH of soybean lipoxidase with emulsified substrates.

Effect of germination on the lipoxidase substrate

It was of interest to determine whether the fatty acid component of the lipoxidase system was altered during germination. Accordingly, the activating influence of octan-2-ol on extracts obtained from pea seeds during various stages of germination was studied. Pea seeds were germinated at 25° for periods up to 6 days, and the catalytic effect on the oxidation of GSH by dialysed extracts prepared

from them was determined before and after addition of octan-2-ol. The reaction was studied in the presence of 0·001 M-KCN to avoid complications due to a cyanide-sensitive reaction which will be referred to later. The results, which are given in

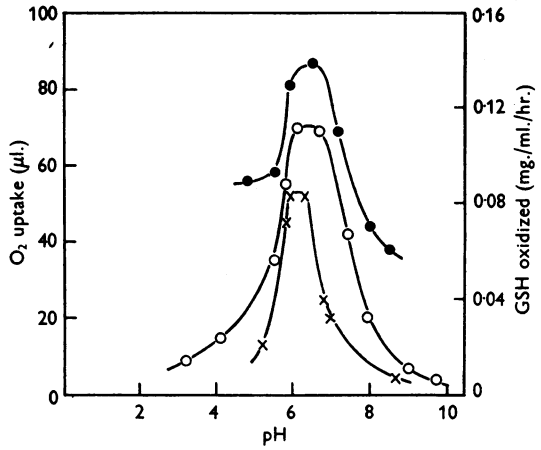

Fig. 6. Effect of pH on the rate of oxidation of GSH and on O_2 uptake by extracts prepared from pea seeds. ●, Green dialysed extract + octan-2-ol + GSH (rate of GSH oxidation); ×, green dialysed extract + octan-2-ol (rate of O_2 uptake); ○, defatted colourless extract + methyl linoleate (rate of O_2 uptake).

Table 1. *Effect of octan-2-ol on the oxidation of GSH by extracts prepared from pea seeds at different stages of germination*

Days of germination	Rate of reaction (mg. GSH oxidized/hr./ml. reaction mixture): Before adding octan-2-ol	Rate of reaction (mg. GSH oxidized/hr./ml. reaction mixture): After adding octan-2-ol	Increase in the rate of reaction due to octan-2-ol (%)
0	0·01	0·10	900
3	0·06	0·20	233
6	0·12	0·12	0

Table 1, showed that as germination progressed there was an increase in the rate of the reaction before the addition of octan-2-ol which was associated with a progressive decrease in the stimulative effect of the alcohol. These results suggest that some change had occurred in the condition of the fatty acid substrate during germination. One possibility is that the fatty acid exists in the ungerminated seeds principally as a part of a complex molecule, which may be broken down either by agents such as octan-2-ol, or by enzymes during germination. Other evidence which is presented later points to the same conclusion. After 6 days germination this process had proceeded to such an extent that the addition of octan-2-ol had little effect on the rate of the reaction.

Location of lipoxidase in the cell

Some experiments were undertaken to determine if the enzyme was associated with the cytoplasmic particles of the cell (mitochondria). Extracts were obtained from pea seeds soaked for 2 days at 20° and separated by fractional centrifuging into mitochondria and supernatant. This was achieved by using the method adopted by Millerd, Bonner, Axelrod & Bandurski (1951).

The lipoxidase activity of the particulate and soluble parts of the cytoplasm were separately determined by measuring the increased O_2 consumption on the addition of methyl linoleate. The enzyme was found to be mainly concentrated in the soluble part of the cytoplasm, the lipoxidase activity of the mitochondria amounting to only 4 % of that of the whole tissue. These tests were repeated with GSH present. The mitochondrial fraction failed to catalyse the oxidation of GSH either before or after the addition of octan-2-ol. A similar negative result was obtained if methyl linoleate was added to mitochondria, either in the presence or absence of octan-2-ol. On the other hand, a positive reaction was obtained when either octan-2-ol or methyl linoleate was added to the supernatant containing the soluble part of the cytoplasm.

The cyanide-sensitive reaction

It has been stated that the oxidation of GSH catalysed by the colourless dialysed extract with methyl linoleate as substrate is cyanide-insensitive. When these experiments were repeated with the green dialysed extract an important difference was noticed. KCN in a concentration of 5×10^{-4} M inhibited by 45 % the rate of oxidation of GSH by the green dialysed extract. This inhibition was not increased by raising the concentration of KCN to 10^{-2} M. These results suggested that two reactions are involved in the oxidation of GSH by the green dialysed extract, one of which is insensitive to cyanide and as shown above appears to be due to a secondary reaction resulting from the activity of a lipoxidase enzyme, while the other is inhibited by cyanide.

Some further experiments were carried out in an attempt to determine the nature of the cyanide-sensitive system. This latter also appears to be dependent on the activity of the lipoxidase enzyme for (1) both cyanide-sensitive and -insensitive reactions require to be activated by the addition of substances like octan-2-ol, and (2) neither the rate nor the absolute amount of O_2 consumed by the green dialysed enzyme, activated by octan-2-ol, was altered by the addition of KCN either in the presence or absence of GSH.

The O_2 consumption of the preparation, therefore, appears to be due to the action of the lipoxidase enzyme and to be independent of GSH. This must mean that the cyanide-sensitive oxidation of GSH like that of the cyanide-insensitive is the result of a secondary reaction coupled with the oxidation of the unsaturated fatty acid.

The rate of oxidation of GSH by the green dialysed extract activated by octan-2-ol was inhibited to the same extent by ethylenediaminetetraacetic acid (Versene), cyanide or azide at a concentration of 0·001 M, suggesting that a metal catalyst is involved in the cyanide-sensitive reaction. Some evidence for the nature of this catalyst was obtained from the following experiment. An enzyme extract was prepared from a pea powder which had previously been defatted by extraction with benzene. This enzyme preparation, with the addition of linoleic acid, catalysed the oxidation of GSH, but the rate of this reaction was not altered by KCN. When linoleic acid was replaced by the benzene extract, the rate of oxidation of GSH was inhibited by KCN (37 % inhibition). The substance present in the benzene extract, which was responsible for this difference, was destroyed by saponification for the only reaction observed after the addition of the unsaponifiable, fatty acid, or aqueous fractions to the enzyme preparation was the cyanide-insensitive oxidation of GSH. If, therefore, a metal catalyst is involved, its activity would appear to be dependent on its association with a lipid material. Clear evidence that this is so must await further investigation.

Holman (1948) has shown that the coupled oxidation of carotenoids occurs as a result of their interruption of the chain oxidation of linoleic acid by reason of their ability to donate hydrogen to an active intermediate. Any substance which furnishes hydrogen atoms should behave likewise, and it seems probable that the coupled oxidation of GSH is due to this cause. The possibility that reaction between the hydroperoxide produced and GSH requires the presence of a metal catalyst could not be excluded. Reactions of this type have been shown to be the cause of the oxidation of thioethers (Robertson, Hartwell & Kornberg, 1944). This interpretation was proved incorrect, however, on finding that GSH was not oxidized when incubated in N_2 in the presence of the green dialysed extract with methyl linoleate containing a high proportion of hydroperoxide. This seems to eliminate the possibility that the cyanide-sensitive oxidation was due to a reaction between the hydroperoxide and the sulphydryl compound.

Mechanism of action of octan-2-ol and similar solvents

The octan-2-ol could not be considered as acting similarly to the butanol used by Morton (1950) for releasing enzymes from cytoplasmic particles, for as shown earlier the addition of methyl linoleate, in the absence of octan-2-ol, to either the green or colourless dialysed extracts, prompted the O_2 uptake and oxidation of GSH to the same extent as that observed when capryl alcohol was added, indicating that the enzyme itself was in an active state in this extract but the substrate was inaccessible to or in a form not acted upon by the enzyme. Hence, in our experiments octan-2-ol appeared to be making a fatty acid available to the enzyme rather than to be activating the enzyme itself.

Results of a similar nature have been reported by Kates (1953), who found that the lecithinase *D* of spinach chloroplasts was activated by ether and other fat solvents. There was no indication from his work, however, whether the action of the fat solvent was exerted on enzyme or substrate.

Bergström & Holman (1948) noted that organic solvents like ether, methyl acetate and acetone, in concentrations of 5 % increased the uptake of oxygen by soybean lipoxidase in an emulsified system; sodium glycocholate in a concentration of 0·2 % behaved likewise. It seems likely that these and other activities, which have been found to increase the activity of the enzyme in emulsion systems (Kies, 1947; Balls, Axelrod & Kies, 1946) do so by their effect on the physical state of the system. Emulsion systems frequently appear to require some surface-active agent to give maximum lipoxidase activity (Holman & Bergström, 1951).

In our work, using methyl linoleate suspended in an aqueous medium as substrate and the colourless dialysed extract as enzyme, the lipoxidase activity was not increased by the addition of octan-2-ol. Clearly the action of octan-2-ol as observed here cannot be explained simply as being due to an alteration in the physical state of the system. The inactivity of surface agents such as bile salts and anti-foam silicone A pointed to the same conclusion. Furthermore, the activitors which have so far been described in the literature merely accelerate a reaction already proceeding; in our experiments no evidence of any enzymic reaction was observed before the addition of octan-2-ol.

The results of experiments designed to determine the influence of concentration of octan-2-ol on the coupled oxidation of GSH showed that a maximum effect of the alcohol was obtained at a concentration of 0·5–1 % (v/v). The reversibility of the action of octan-2-ol was demonstrated by finding that removal of the alcohol by prolonged dialysis led to inactivation; no destruction of the enzyme occurred during this treatment since the further addition of octan-2-ol produced a rapid lipoxidase reaction.

It was thought possible that the effect of octan-2-ol and other fat solvents might be due to their action in dissolving fat-soluble components of the

cell. The ability of various solvents to activate the enzyme system could not, however, be correlated with their ability to extract either fats or phospholipids from pea powder or aqueous extracts of the same tissue. The extracts obtained were used to determine (1) total fat-soluble components, and (2) the phospholipids as estimated by determination of the total phosphorus content. The results obtained in these experiments are shown in Table 2.

Table 2. *Total soluble matter and total phosphorus content in extracts prepared from pea powders*

Solvent	Relative activity in enzyme system	Extraction from green dialysed extract: Total soluble matter (mg./100 ml.)	Extraction from green dialysed extract: Phosphorus (mg./100 ml.)	Extraction from pea powder: Total soluble matter (mg./100 g.)	Extraction from pea powder: Phosphorus (mg./100 g.)
Octan-2-ol	100	956	2·4	1680	14·88
Benzene	30	100	0·67	1050	14·60
n-Butanol	50	265	9·6	1000	9·6
Light petroleum	5	20	0·13	960	6·4
n-Propanol	0	232	16·00	1782	14·64
*iso*Butanol	2	264	9·6	1000	9·6

These negative results prompted us to investigate the relative effectiveness of different alcohols and other fat solvents. The solvents were added in a concentration of 0·01 ml./ml. and their relative effectiveness was calculated by comparing the rate of the reaction with that induced by octan-2-ol. The results obtained are given in Table 3.

The fact that some fat solvents were not capable of activating the enzyme extract was not due to any specific inhibitory action on the enzyme itself. This was shown by dissolving methyl linoleate in such solvents as light petroleum or *iso*butanol; on the addition of such solutions to the colourless dialysed extract there was a vigorous oxygen uptake and oxidation of GSH.

The low potency of the non-polar fat solvents compared with polar solvents such as certain alcohols is noticeable. With these latter, although the data are insufficient to draw any firm conclusions, there appears to be some relation between structure of the alcohol and its ability to activate the reaction. Primary alcohols with hydrocarbon chains of 3 or more carbon atoms were effective, the effectiveness tending to increase with increase in length of the carbon chain; very long chain alcohols such as hexadecan-1-ol were, however, ineffective. Branching of the chain had no pronounced effect. Substitution of hydrogen on the carbinol group by methyl reduced the effectiveness of the alcohol. Secondary alcohols were less effective than primary, and tertiary even less than secondary. The effect of substitution by a methyl group on the carbinol group was reversed to some extent by the presence of a long hydrocarbon chain attached to this group (cf. octan-2-ol with pentan-2-ol and hexan-2-ol). It is well known that the ability of a molecule to orientate at an aqueous hydrocarbon interface usually depends on the possession of well separated polar and non-polar groups, and it seems probable that the activity of the different alcohols may be explicable on this basis.

DISCUSSION

Perhaps the most interesting observation made during the course of this work was the ability of small amounts of the higher alcohols to initiate an enzymic reaction. As shown in this paper the action of the alcohol seems to be due to its ability to change the condition of a fatty acid substrate, thus making it available to the enzyme. It is tempting to assume that the fatty acid is either adsorbed on or associated as a complex with other substances in the tissue, and that this association is broken by the alcohol. The substances most likely to be involved are proteins, and in this respect the work of Kendall (1941) and of Cohn, Hughes & Weare (1947) is of interest. These workers have shown that albumins even after repeated crystallization still contain 0·5 % of lipid material. This lipid material has been shown to be mainly fatty acid and is extremely difficult to remove from the protein; in particular these complexes are not resolved by crystallization. Luck (1949) has summarized the evidence showing that fatty acid anions may be bound to albumins and has discussed the electrostatic forces involved.

The ability of albumins to bind other large anions has also been stressed by many workers (Grollman, 1925; Bennhold, 1932; Fairley, 1941; Kendall, 1941), and has since been confirmed by Cohn *et al.* (1947) for purer specimens of serum albumin. Of especial interest, in relation to our work, was the observation of these latter workers that long-chain alcohols are readily bound by this protein. These alcohols form complexes with the albumin and are carried by them through repeated crystallizations. The effectiveness with which these alcohols formed complexes with the proteins increased with the

Table 3. *The activating effect of different alcohols and other fat solvents on the coupled oxidation of GSH by a lipoxidase system*

Solvent	Formula	Rate of reaction (mg. GSH oxidized/ml. extract/hr.)	Relative* activity	Solubility (g./100 ml. water)
Primary alcohols				
Methanol	CH_3OH	0	0	Very soluble
Ethanol	$CH_3.CH_2OH$	0	0	Very soluble
Propan-1-ol	$CH_3.CH_2.CH_2OH$	0	0	Very soluble
Butan-1-ol	$CH_3.(CH_2)_2.CH_2OH$	0·1	50	7·9
*iso*Butan-1-ol	$(CH_3)_2CH.CH_2OH$	0·004	2	9·5
Pentan-1-ol	$CH_3.(CH_2)_3.CH_2OH$	0·092	46	2·7
Hexan-1-ol	$CH_3.(CH_2)_4.CH_2OH$	0·25	125	0·59
2-Ethylbutan-1-ol	$(CH_3CH_2)_2CH.CH_2OH$	0·21	105	0·63
Heptan-1-ol	$CH_3.(CH_2)_5.CH_2OH$	0·13	156	0·09
2-Ethylhexan-1-ol	$CH_3.(CH_2)_3.CH(CH_3CH_2).CH_2OH$	0·28	140	0·07
3:5:5-Trimethylhexan-1-ol	$CH_3.C(CH_3).CH_2.CH(CH_3).CH_2.CH_2OH$	0·175	87	Very slightly soluble
Nonan-1-ol	$CH_3.(CH_2)_7.CH_2OH$	0·425	215	Insoluble
Decan-1-ol	$CH_3.(CH_2)_8.CH_2OH$	0·45	225	Insoluble
Hexadecan-1-ol	$CH_3.(CH_2)_{14}.CH_2OH$	0	0	Insoluble
Secondary alcohols				
*iso*Propanol	$(CH_3)_2CHOH$	0	0	Very soluble
Pentan-2-ol	$CH_3.(CH_2)_2.CH(CH_3)OH$	0·039	20	5·3
Hexan-2-ol	$CH_3.(CH_2)_3.CH(CH_3)OH$	0·048	24	Very slightly soluble
Heptan-4-ol	$(CH_3CH_2)_2CHOH$	0·166	83	Insoluble
Octan-2-ol	$CH_3(CH_2)_5.CH(CH_3)OH$	0·2	100	Insoluble
Tertiary alcohols				
2-Methylbutan-2-ol	$(CH_3)_2C(CH_3CH_2).OH$	0·019	10	12·5
2-Methylpentan-2-ol	$CH_3.CH_2.CH_2.C(CH_3).OH$	0·048	24	Slightly soluble
Other fat solvents				
Light petroleum (b.p. 40–60°)		0·01	5	—
Ether	$C_2H_5.O.C_2H_5$	0	0	7·5
Chloroform	$CHCl_3$	0·01	5	1·0
Acetone	$CH_3.CO.CH_3$	0·004	2	Very soluble
Benzene	C_6H_6	0·06	30	0·08
Toluene	$C_6H_5.CH_3$	0·06	30	0·047

* The activity when octan-2-ol was used was taken as 100.

length of the carbon chain at least up to decanol; long-chain alcohols such as octadecan-1-ol or hexadecan-1-ol were ineffective. These results are very similar to ours, and we may therefore speculate whether the activity of the alcohols recorded here is due to their ability to be adsorbed on or form complexes with a protein and thereby displace other large anions from its surface. A more detailed study along these lines will be necessary before any conclusions can be drawn as to the mechanism of the action of these alcohols.

SUMMARY

1. Under certain conditions a rapid oxidation of glutathione (GSH) was observed when it was added to a dialysed extract prepared from ungerminated peas. The reaction was found to be due to a coupled oxidation of the sulphydryl compound and an unsaturated fatty acid of the linoleic acid type under the action of a lipoxidase enzyme.

2. Extracts from ungerminated pea seeds showed no evidence of any lipoxidase activity as judged by oxygen consumption or ability to oxidize GSH. The enzymic activity of the extract could be initiated by the addition of small amounts of certain alcohols. The alcohols have been shown to act by altering the condition of the fatty acid substrate of the lipoxidase in such a way that it is made accessible to the enzyme.

3. During germination, changes occur in the fatty acid substrate in pea seeds similar to those induced by the action of the alcohols.

4. The relation between chemical structure of the alcohols and their ability to initiate the lipoxidase reaction was studied and the mechanism of their action discussed.

5. The lipoxidase enzyme and substrate were found to be associated with the soluble part of the cytoplasm, and to be absent from the mitochondria.

6. The coupled oxidation of GSH was found to be the result of two reactions, one of which was cyanide-sensitive and the other cyanide-insensitive. There is evidence that in the former reaction a metal catalyst is involved.

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