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THE OXIDATION OF CAROTENOIDS BY MITOCHONDRIA FROM SUGAR BEET LEAVES—III.

CROCIN OXIDATION BY A PEROXIDASE SYSTEM

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Abstract—The crocin-destroying system retained in the residue following treatment of mitochondrial preparations from sugar beet leaves with Triton-X-100 does not fall within the lipid-requiring category of catalysts which oxidize carotenoids. The system is similar to indole-acetic acid oxidase of plant tissues with respect to its peroxidase content and reaction with phenolic compounds. The residue contains an indole-acetic acid oxidase which is optimally active at the pH optimum for crocin destruction (pH 3·6); both activities are stimulated by 2,4-dichlorophenol. Catalase present in the preparation appears to have no influence on crocin-destroying activity. Generation of hydrogen peroxide was not detected, although there is an absolute requirement for oxygen. The soluble fraction of the leaf contains a thermolabile factor which stimulates crocin destruction by the particulate system. Partial purification and resolution of this factor into several fractions by ion-exchange chromatography is accompanied by purification and fractionation of peroxidase activity. It is concluded that crocin is destroyed by an aerobic oxidation mediated by peroxidase, although the exact requirements for the reaction have not been established. Evidence is presented to support the claim that the particulate system participates in the loss of carotene which occurs when sugar beet leaves are damaged.

INTRODUCTION

IT WAS reported in an earlier paper in the series[1] that treatment of mitochondrial preparations from sugar beet leaves with Triton-X-100 yields an extract and residue, both of which catalyse the oxidation of crocin, the digentiobiose ester of crocetin. Evidence was presented to show that the two fractions contain different systems; activity in the residue reflects that of untreated particles, whereas extracted activity, for which an unsaturated fat oxidase system appears to be responsible,[2] is not operative prior to treatment of the particles with detergent.

The residue system, which shows maximal activity at pH 3·6, is inhibited by cyanide and stimulated by 8-hydroxyquinoline, and in particular, is stimulated by the cell soluble fraction which, itself, has no oxidizing activity towards crocin.

The present paper describes the properties of the residue in more detail and it is shown that crocin destruction by the residue is not coupled to unsaturated fat oxidation, but is mediated by a system which resembles the peroxidase-containing indole-acetic acid oxidase of many plant tissues.[3] A preliminary report of this work has been presented.[4]

RESULTS AND DISCUSSION

Peroxidase Activity of Washed Residue

During a comparative study of the properties of the extract and residue obtained after treatment of mitochondria with Triton-X-100, the distribution of peroxidase activity between

[1] J. FRIEND and J. W. DICKS, *Phytochem.* **5**, 205 (1966).
[2] J. W. DICKS and J. FRIEND, *Phytochem.* **6**, 1193 (1967).
[3] R. C. HARE, *Bot. Rev.* **30**, 192 (1964).
[4] J. W. DICKS and J. FRIEND, *Biochem. J.* **99**, 38P (1966).

the derivative fractions was determined. The results, presented in Table 1, indicate that there is no loss in yield and that the bulk of peroxidase activity is retained in the residue. It is interesting to note that the increase in specific activity of peroxidase between the washed residue and the mitochondria suspended in triton (2·26-fold) is very similar to that observed for crocin-destroying activity between similar fractions from a different preparation (2·21-fold). This observation indicated the possibility that peroxidase, in some way, participates in crocin destruction by the washed residue.

TABLE 1. DISTRIBUTION OF PEROXIDASE ACTIVITY IN FRACTIONS DERIVED FROM MITOCHONDRIA BY TREATMENT WITH TRITON-X-100

	Peroxidase activity				Crocin* destruction specific activity (relative to T)
			Specific activity		
	Activity (units/ml)†	Protein (mg/ml)	(Units/mg protein)	(Relative to T)	
Particles suspended in 1 % Triton (T)	5·8	4·1	1·41	1·00	1·00
Supernatant from T after centrifugation (E_t)	0·8	2·1	0·38	0·27	1·88
Residue from T after centrifugation, suspended in buffer after washing (R_w)	5·1	1·6	3·19	2·26	2·21

* The results for crocin destruction were obtained with similar fractions from a different preparation to those employed for the peroxidase determinations. See Table 5 of the first paper in this series.[1]

† For definition of a unit of peroxidase activity and special conditions of peroxidase assay used in this experiment, see Experimental.

The Effect of Acetone Treatment, and of Linoleic Acid on the Crocin-destroying Activity in the Washed Residue

It has already been shown that the crocin-destroying activity of Triton extracts of mitochondrial preparations appears to depend on a haemoprotein-type unsaturated fat oxidase.[2] Despite a number of well-defined differences between the activity of extract and residue,[1] the finding that the residue contains peroxidase (which has a protohaem prosthetic group) made it necessary to determine whether unsaturated fat oxidase activity could also account for crocin destruction by the residue.

Treatment of a washed residue preparation (R_w) with chilled acetone produced an intractable gummy material which adhered to the vessel walls and glass stirring-rod. The aqueous acetone was decanted and centrifuged, yielding a pellet and a clear supernatant ($R_{ac}s$). Since the pellet ($R_{ac}p$) contained about 20 per cent of the protein and between 20 and 25 per cent of the original activity, no significant change in specific activity was observed (Table 2). Fraction $R_{ac}s$, extracted by acetone, was inactive towards crocin on its own or when it was added to $R_{ac}p$. Slightly peroxidized linoleic acid slightly inhibited the activity of the untreated residue (R_w), and substantially inhibited the activity of $R_{ac}p$.

These results are in clear contrast to those obtained by similar treatment of Triton extracts,[2] and thus confirm the earlier conclusion[1] that neither unsaturated fat oxidase nor

lipoperoxidase[5] activity is significantly involved in crocin oxidation by the particulate system under investigation.

TABLE 2. THE EFFECT OF ACETONE TREATMENT AND OF LINOLEIC ACID ON THE CROCIN-DESTROYING ACTIVITY OF A WASHED RESIDUE PREPARATION

Additions	Crocin destruction		Protein (mg)	Specific activity (mμmoles crocin/mg. protein in 20 min)
	mμmoles in 20 min	% of R_w		
Washed residue (R_w)	31·3	100	0·38	82·4
R_w + linoleic acid*	29·9	96	0·38	78·7
Precipitate derived from R_w by acetone treatment ($R_{ac}p$)	7·5	24	0·08	93·7
Supernatant fraction derived from R_w by acetone treatment ($R_{ac}s$)	0	0	0	—
$R_{ac}p + R_{ac}s$	7·5	24	0·08	93·7
$R_{ac}p$ + linoleic acid*	2·7	9	0·08	33·7

* Linoleic acid, containing approximately 1 % hydroperoxides, was added at a final concentration of 0·32 mM.

The Effects of Phenolic Compounds and other IAA-oxidase modifiers on the Crocin-destroying Activity in the Washed Residue

The stimulation by 8-hydroxyquinoline of crocin destruction catalysed both by mitochondrial suspensions and washed residue preparations was not satisfactorily explained in our earlier studies on the basis of its acting as a metal chelator.[1] The possibility that stimulation may be related to the phenolic nature of this compound was therefore tested by determining the effect of a range of phenols on the activity of a washed residue (Table 3). The diversity of responses observed is reflected in the classification of the phenolic compounds employed in this study into three groups according to their effects at two concentrations.

Although a unifying relationship between structure and action of the phenols tested is not obvious, some points of comparison may be noted. Most of the stimulatory monohydric phenols possess another substituted group in the *para* position. The possible importance of *para* substitution is emphasized by comparing the effects of the *para*- and *ortho*-hydroxy cinnamic acids (coumaric acids) and β-naphthol with α-naphthol. The complexity of interaction is underlined by examining the effects of compounds structurally related to cinnamic acid. The *p*-hydroxy derivative (*p*-coumaric acid) is placed in Group 1 (Table 3), exerting a powerful stimulatory effect. The presence of a hydroxy- or methoxy-group *ortho* to the original hydroxy-group (caffeic and ferulic acids, respectively) confers drastically different properties upon the derivatives; they exert a small stimulatory effect at a concentration of 50 μM, but a tenfold increase in their concentration results in considerable inhibition (Group 2, Table 3). Sinapic acid, which has a second methoxy-group, also *ortho* to the hydroxy-group, displays a third type of behaviour, for it is an extremely effective inhibitor at both concentrations (group 3, Table 3).

[5] J. A. BLAIN and E. C. C. STYLES, *Nature* **184**, 1141 (1959).

TABLE 3. THE EFFECTS OF VARIOUS PHENOLIC COMPOUNDS ON CROCIN DESTRUCTION CATALYSED BY A WASHED RESIDUE PREPARATION

Phenolic compound	% Stimulation (+) or inhibition (−) at a concentration of	
	50 μM	500 μM
Group 1		
Vanillin	+148	+190
β-Naphthol	+130	+135
p-Coumaric acid	+107	+121
2,4-Dichlorophenol	+86	+>200*
2,4-Dibromophenol	+77	+189
2,4-Diiodophenol	+45	+115
Na *p*-Aminosalicylate	+39	+>200*
Chlorogenic acid	+53	+97
8-Hydroxyquinoline	+35	+74
o-Coumaric acid	+6	+59
p-Hydroxybenzoic acid	Ineffective	+20
Group 2		
Ferulic acid	+9	−70
Caffeic acid	+14	−41
Group 3		
Sinapic acid	−80	−90
Pyrogallol	−27	−97
α-Naphthol	−26	−83
Catechol	Ineffective	−26

* In these cases, crocin destruction is complete within the incubation period (20 min).

The peroxidase-catalysed oxidation of metabolically important compounds, such as indole-acetic acid[6] and reduced pyridine nucleotides[7] are similarly influenced by phenolic compounds. As a generalized statement, where phenols and peroxidase interact, monohydric phenols are stimulatory, whereas dihydric and polyhydric phenols are inhibitory. A reasonable explanation of this interaction is provided by Yamazaki's free radical theory of peroxidase reactions.[8] Hydrogen donors which influence the oxidation by peroxidase of dihydroxyfumarate and triose reductone were shown to fall into two groups. According to Yamazaki's concept, inhibitory (redogenic) donors are oxidized to stable products by two one-electron steps ($AH_2 \rightleftharpoons AH^{\bullet} \rightleftharpoons A$). In contrast, stimulatory (oxidogenic) donors were considered to yield radicals ($AH^{\bullet}$) lacking the ability to undergo a second one-electron oxidation (to give A), but capable of either being reduced back to AH_2 at the expense of a secondary donor (RH), or to give polymerization products by self-condensation.

In terms of the effect of these two types of donor upon peroxidase-catalysed oxidations, Yamazaki's designation delineates those donors which can fulfil a cofactor role by cyclic oxidation and reduction (oxidogenic) and those which inhibit competitively (redogenic). Collectively, the data presented in Table 3 support the hypothesis that peroxidase is responsible for the crocin-destroying activity of washed residue preparations.

[6] R. H. KENTEN, *Biochem. J.* **59**, 110 (1955).
[7] T. AKAZAWA and E. E. CONN, *J. Biol. Chem.* **232**, 403 (1958).
[8] I. YAMAZAKI, *Proc. Int. Symposium Enzyme Chemistry*, p. 224, Tokyo (1958).

The exceptional behaviour of ferulic and caffeic acids is most interesting in the light of a report[9] that ferulic acid acts as a "coenzyme" for indole-acetic acid oxidase of pineapple at low concentrations, but is inhibitory at higher concentrations in the presence of a more potent cofactor *p*-coumaric acid. In view of this correlation, the effects of further modifiers of indole-

TABLE 4. THE EFFECTS OF SOME IAA-OXIDASE MODIFIERS AND OTHER PEROXIDASE SUBSTRATES ON CROCIN DESTRUCTION CATALYSED BY THE PARTICULATE SYSTEM

Reagent and concentration (μM)	% Stimulation (+) or inhibition (−) of crocin destruction catalysed by	
	M*	R_w*
Chlorogenic acid		
10	+21	
50		+53
100	+35	
500		+97
1000	+110	
Scopoletin		
10	+10	
100	+33	
1000	+63	
β-Methyl umbelliferone		
50		+9
100	+22	
500		+60
1000	+70	
Maleic hydrazide		
100	0	
500		+7
1000	+9	
5000	+18	
Indole acetic acid		
10	−16	
50		−19
100	−24	
500		−29
1000	−41	
Reduced nicotinamide adenine dinucleotide		
50		−38
500		−80

* M: mitochondrial suspension. R_w: washed residue preparation.

acetic acid oxidase activity on crocin destruction by the particulate system were determined (Table 4).

Stimulation by β-methyl umbelliferone is a feature shared by some preparations of indole-acetic acid oxidase. It has been suggested[3] that this coumarin derivative exerts its

[9] W. A. GORTNER and M. J. KENT, *J. Biol. Chem.* **233**, 731 (1958).

influence by suppressing the activity of endogenous inhibitors, but maleic hydrazide, which is considered to act in a similar manner, has little effect on crocin destruction, even at a concentration as high as 5 mM.

Of the dihydric phenols originally tested with washed residue preparations (Table 3), only chlorogenic acid was found to be stimulatory. When this experiment was repeated with a mitochondrial preparation, a similar result was obtained. Scopoletin also stimulates. The effect of these two compounds is difficult to interpret, since they are both potent inhibitors of indole-acetic acid oxidation catalysed either by horse-radish peroxidase[10] or by IAA oxidase from several plant sources.[9,11].

The inhibition by indole-acetic acid and reduced nicotinamide adenine dinucleotide may be related to the fact that they are substrates for peroxidase.[6,7] The effects of their addition render the possibility that crocin destruction is coupled to the endogenous oxidation of either of these compounds most unlikely.

Indole-Acetic Acid Oxidase Activity of Particulate Preparations

Washed residue is capable of degrading indole-acetic acid without the addition of cofactors (Fig. 1). Differences in the procedure for estimating crocin- and indole-acetic acid-

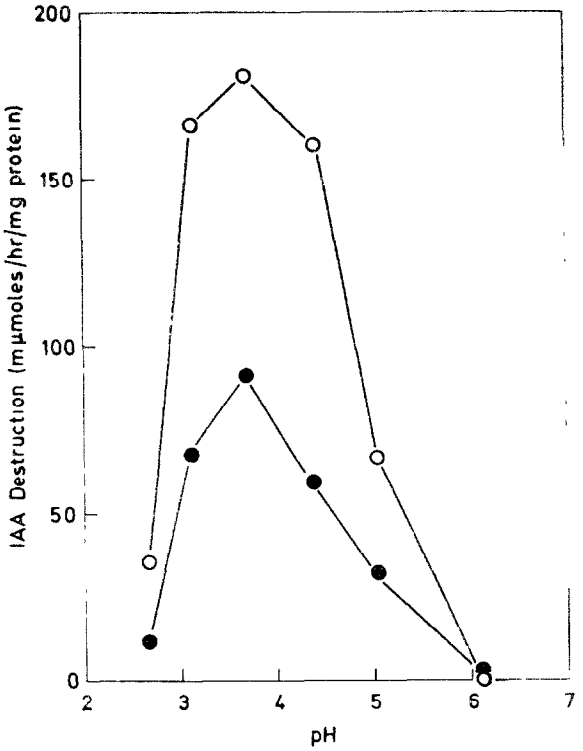

FIG. 1. pH-ACTIVITY RELATIONSHIP FOR INDOLE-ACETIC ACID OXIDASE OF A WASHED RESIDUE PREPARATION.
Closed circles: without 2,4-dichlorophenol.
Open circles: with 100 μM 2,4-dichlorophenol.

[10] G. W. SCHAEFFER, J. G. BUTA and F. SHARPE, *Physiol. Plant.* **20**, 342 (1967).
[11] W. A. ANDREAE, *Nature* **170**, 83 (1952); R. S. RABIN and R. M. KLEIN, *Arch. Biochem. Biophys.* **70**, 11 (1957); L. SEQUEIRA, *Phytopath.* **54**, 1078 (1964).

oxidase activities prevent a strict comparison between rates of oxidation of these two substrates, but from calculations based on results obtained with the same washed residue preparation, it appears that they are oxidized at a similar rate (52 mμmoles crocin/mg protein in 20 min at pH 3·7 at an initial substrate concentration of 70 μM; compared with 91 mμmoles indole-acetic acid/mg protein in 60 min at an initial substrate concentration of 286 μM). It would appear significant that the reaction is stimulated by 2,4-dichlorophenol, a feature it shares both with other indole-acetic acid oxidases and with the crocin-destroying system. Furthermore, the pH optima for destruction of crocin and indole-acetic acid by the particulate system are identical. Reported pH optima for indole-acetic acid oxidases vary with the sources of enzyme and methods of assay, but the enzymes from *Omphalia*[12] and pineapple[9] exhibit maximal activity at pH 3·5 and pH 4·0 respectively.

The Effect of Manganese on Crocin Destruction by the Particulate System

Since manganous ions generally activate or stimulate aerobic oxidations catalysed by peroxidase (for review, see Ref. 13), the effects of various concentrations of manganese on

TABLE 5. THE EFFECT OF MANGANESE ON CROCIN DESTRUCTION BY UNDIALYSED AND DIALYSED MITOCHONDRIAL SUSPENSIONS

Treatment*	Buffer	mμmoles crocin destroyed in 20 min at Mn^{2+} concentration of: 0	10	100	1000 (μM)
1	Acetate	48·5	40·7 (16)†	35·9 (26)	34·2 (30)
1	Phosphate-citrate	48·8	41·8 (14)	30·7 (37)	18·3 (63)
2	Phosphate-citrate	33·7	27·3 (19)	19·2 (43)	12·7 (62)
3	Phosphate-citrate	36·9	31·8 (14)	25·8 (30)	17·0 (54)
4	Phosphate citrate	32·5	28·6 (11)	23·4 (28)	11·4 (64)

* Treatments: None (1); dialysis for 15 hr at 2° against 5 mM pH 7·0 phosphate buffer (2), containing 1 mM 8-hydroxyquinoline (3), containing 1 mM EDTA (4).
† Figures in brackets refer to % inhibition by Mn^{2+} at the specified concentration.

crocin destruction catalysed by the particulate system were determined (Table 5). No stimulation of activity was observed. The metal ion had insignificant effect when present at 1 μM, but at progressively higher concentrations it exerted an increasingly inhibitory effect. Dialysis of the mitochondrial suspension against dilute buffer, against EDTA and against 8-hydroxyquinoline resulted in some loss of activity in each case. However, this loss was not reversed by manganese. The levels of inhibition by manganese in the dialysed samples were

[12] P. M. RAY and K. V. THIMANN, *Arch. Biochem. Biophys.* **64**, 175 (1956).
[13] B. C. SAUNDERS, A. G. HOLMES-SIEDLE and B. P. STARK, *Peroxidase. The Properties and Uses of a Versatile Enzyme and of Some Related Catalysts*, Butterworths, London (1964).

similar to those in the undialysed preparation, and it is clear that losses of activity accompanying dialysis, which is not dependent on the presence of a metal-chelating agent in the dialysing medium, cannot be explained by removal of endogenous manganese.

The inhibition by manganese of *Lupinus album* indole-acetic acid oxidase in citrate buffers was explained in terms of the formation of an inactive enzyme-manganese-citrate complex.[14] In the present work, citrate-phosphate buffers have been used routinely. When acetate buffer was employed, manganese still inhibited crocin destruction, although less effectively at 1 mM than in citrate-phosphate buffer (Table 5). An alternative interpretation of manganese inhibition is provided by the studies of Hillman and Galston,[15a] who suggested that inhibition of pea indole-acetic acid oxidase by manganese in the presence of very low concentrations of 2,4-dichlorophenol was a result of imbalance in the manganese/phenolic cofactor ratio at low endogenous phenol concentrations.

Chelation of manganese by citrate anions has been suggested as an explanation for the low pH optimum observed for indole-acetic acid oxidases assayed in citrate buffers.[15b] However, since there appears to be no requirement for manganese by the particulate crocin-destroying system in sugar beet, it is unlikely that this explanation can account for the low pH optimum.

Investigation of the Requirement for Hydrogen Peroxide

Goldacre and Galston[16] have shown that a number of substituted monohydric phenols, particularly 2,4-dichlorophenol, are potent and relatively specific inhibitors of catalase. Indeed, the stimulatory effect of 2,4-dichlorophenol on indole-acetic acid oxidase activity of pea tissue was thought initially to be due to a peroxide-sparing effect by inhibition of catalase present as a contaminant in the enzyme preparation,[17] although it was concluded later that the phenol acted in a different way when it was demonstrated that catalase-free preparations could still be activated by the phenol.[18]

Thus, the possibility that the stimulation by monohydric phenols of crocin destruction by the particulate system could be explained by a peroxide-sparing mechanism through inhibition of catalase had to be considered. It was demonstrated that residue preparations contained a thermolabile catalyst which destroyed hydrogen peroxide in a reaction exhibiting first-order kinetics ($\log A_0/A$ vs. time gave a straight line). However, at a concentration of 2,4-dichlorophenol (100 μM) which is sufficient to inhibit catalase completely,[16] the catalytic activity of the residue was inhibited by only 30 per cent. It may be that the phenol is converted to an inactive product during its preincubation with a relatively crude preparation prior to the addition of peroxide in the assay, and its effective concentration lowered.

Investigation of the pH-activity relationship for particulate catalase revealed that activity decreased sharply below pH 5·0 (Fig. 2), an observation which is in agreement with data on catalase from other plant sources.[19] Since crocin destruction by the particulate system is maximal at pH 3·6, it is unlikely that catalase is an effective endogenous inhibitor of the system.

By lowering the pH of a residue preparation to a value of 3·6 with citric acid, and main-

[14] R. E. STUTZ, *Plant Physiol.* **32**, 31 (1957).
[15a] W. S. HILLMAN and A. W. GALSTON, *Physiol. Plant.* **9**, 230 (1956).
[15b] J. B. MUDD, B. G. JOHNSON, R. H. BURRIS and K. P. BUCHHOLTZ, *Plant Physiol.* **34**, 144 (1959).
[16] P. L. GOLDACRE and A. W. GALSTON, *Arch. Biochem. Biophys.* **43**, 169 (1953).
[17] A. W. GALSTON, J. BONNER and R. S. BAKER, *Arch. Biochem. Biophys.* **42**, 456 (1953).
[18] P. L. GOLDACRE, A. W. GALSTON and R. L. WEINTRAUB, *Arch. Biochem. Biophys.* **43**, 358 (1953).
[19] A. W. GALSTON, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 2, p. 789, Academic Press, New York (1955).

taining a temperature of 30° for 1 min, the preparation could be separated into a sedimentable precipitate and a clear supernatant. Both fractions were capable of catalysing crocin destruction (Table 6). Although catalase was completely inactivated by this treatment, the

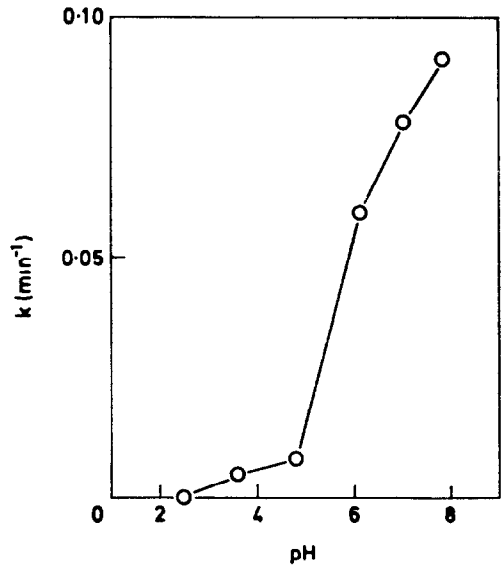

FIG. 2. pH-ACTIVITY RELATIONSHIP FOR PARTICULATE CATALASE.
A mitochondrial preparation was employed in this experiment.

TABLE 6. THE EFFECT OF EXPOSURE TO LOWERED pH AND ELEVATED TEMPERATURE ON CROCIN-DESTROYING AND CATALATIC ACTIVITIES OF A WASHED RESIDUE PREPARATION

Fraction	mμmoles crocin destroyed in 20 min		% stimulation by 2,4-DCP	Catalase activity (k (min^{-1}))
	−2,4-DCP	+2,4-DCP*		
Washed residue (R_w)	45·4	91·2	102	0·114
Precipitate obtained by pH/temp. treatment	21·1	34·6	64	0
Supernatant obtained by pH/temp. treatment	17·1	43·4	153	0

* 2,4-Dichlorophenol was added at a final concentration of 100 μM.

crocin-destroying activity in each fraction was stimulated by the addition of 2,4-dichlorophenol. This experiment strongly suggests that catalase participates neither as an inhibitor nor as a catalyst of crocin destruction in the particulate system. In view of these results it was not surprising to find that the addition of excess quantities of commercial catalase had an insignificant effect on crocin destruction by the residue.

Hydrogen peroxide is commonly generated in living tissues by the action of numerous flavoprotein oxidases. In our earlier studies,[1] we failed to demonstrate any significant stimulation of crocin destruction catalysed by mitochondria by the addition of substrates for a range of flavoprotein oxidases. In the present work, it has not been possible to detect peroxide generation in the washed residue either by the sensitive colorimetric method in which peroxides oxidize ferrous thiocyanate to the red ferric salt, or by replacing peroxide with residue in a peroxidase assay using guaiacol as hydrogen donor.

Although crocin destruction is readily catalysed by peroxidase in the presence of hydrogen peroxide generated enzymatically by the action of glucose oxidase on glucose, the rate of reaction is unaffected by the addition of *p*-coumaric acid (see Experimental), one of the

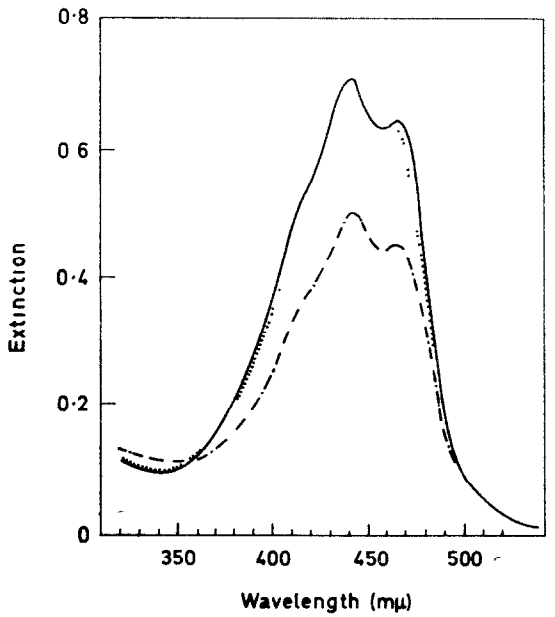

FIG. 3. VISIBLE ABSORPTION SPECTRUM OF CROCIN IN WATER, AND OF WATER-SOLUBLE COMPONENTS OF THE REACTION MIXTURE AFTER INCUBATION OF CROCIN WITH A WASHED RESIDUE PREPARATION UNDER AEROBIC AND ANAEROBIC CONDITIONS.

Substrate in aqueous solution (solid line); water-soluble components of reaction mixture at 15 min incubation with washed residue under aerobic (dashed line); and anaerobic (dotted line) conditions.

monophenols which stimulate the activity of the particulate system (Table 3). Thus, the absolute requirement for oxygen by the particulate system (Fig. 3) cannot be ascribed to a dependance on peroxide generation, and it is tentatively suggested that the reactivity of particulate preparations towards crocin is an example of the aerobic oxidations which can be mediated by peroxidase. However, since peroxidase alone in the presence of oxygen is incapable of catalysing the oxidation of crocin the requirement for an unknown cofactor in the particles must be postulated.

Interaction of the Particulate System with Components of the Cell Soluble Fraction

Although the supernatant (cell soluble fraction) obtained by centrifugal removal of mitochondria from leaf homogenates possesses insignificant crocin-destroying activity, it enhances

the activity of the particulate system.[1] The stimulatory effect of the cell soluble fraction varies from preparation to preparation and depends on relative contributions of a heat-labile stimulatory component and heat-stable inhibitory compounds. The results recorded in Table 7 illustrate the existence of thermostable and, in part, diffusible inhibitors and are typical of the extreme situation in which the nett stimulation is very low.

TABLE 7. EVIDENCE FOR THE DUAL NATURE OF THE CELL SOLUBLE FRACTION IN ITS EFFECT ON CROCIN DESTRUCTION CATALYSED BY A MITOCHONDRIAL SUSPENSION

Additions	mμmoles crocin destroyed in 20 min	% stimulation (+) or inhibition (−)
None (control)	33·6	—
Untreated cell soluble fraction (S)	35·2	+5
Dialysed S*	45·6	+36
Boiled S	21·3	−37
Boiled, dialysed S†	27·2	−19

* Dialysed against 100 vol 5 mM tris-HCl buffer (pH 7·8) at 2° for 3 hr.

† Dialysis preceded boiling.

Protein concentration in reaction flasks: 0·6 mg (mitochondrial suspension) and 0·4 mg (cell soluble fraction) in 2 ml.

A clue to the nature of the stimulatory component was obtained whilst investigating the possibility that a haemoprotein may be responsible for the unsaturated fat oxidase activity of the Triton-extract system.[2] The effects of cytochrome *c* and peroxidase, as representative haemoproteins, on crocin destruction by the Triton extract was determined, and their effects on an untreated mitochondrial preparation and a washed residue were determined for comparison (Table 8).

TABLE 8. THE EFFECTS OF PEROXIDASE AND CYTOCHROME *c* ON CROCIN DESTRUCTION CATALYSED BY FRACTIONS DERIVED FROM MITOCHONDRIA BY TREATMENT WITH TRITON-X-100

Additions†	mμmoles crocin destroyed in 20 min by*: M	R_w	E_t
None (controls)	41·9	28·8	54·4
Peroxidase	95.7	112·0	71·5
Cytochrome *c*	60.3	41·3	89·3
		% stimulation	
Peroxidase	128	288	31
Cytochrome *c*	44	43	64
% Stimulation by peroxidase / % Stimulation by cytochrome *c*	2·9	6·7	0·5

* M: mitochondrial suspension, R_w: washed residue, E_t: Triton extract.

† Haemoprotein additions: 100 μg peroxidase (Koch-Light, RZ 0·6) or 120 μg cytochrome *c* (BDH, 60–70 per cent).

On a weight for weight basis, the greater effectiveness of cytochrome *c* over peroxidase in stimulating crocin destruction by the Triton extract (Et) can be correlated with the higher capacity of cytochrome *c* to catalyse crocin destruction in the presence of acetone-soluble components of the detergent extract,[2] and the results were in agreement with the hypothesis that both haemoproteins, added to Triton extracts, are acting as unsaturated fat oxidases. In contrast, the effect of these haemoproteins on the activity of the particulate system point to a specific action of peroxidase, for which cytochrome *c* is a moderately effective substitute.

Partial purification and separation of the soluble stimulatory component into several fractions has been effected by a procedure which included dialysis, treatment with salmine sulphate, ammonium sulphate and acetone, and finally chromatography on DEAE cellulose. The results of this investigation (unpublished) suggest that multiple components of peroxidase are responsible for the stimulation of crocin destruction afforded by cell soluble fraction.

Endogenous Inhibitors

Two of the fractions obtained by ion-exchange chromatography in the procedure mentioned in the previous section of the paper have been shown to inhibit crocin destruction catalysed by the particulate system. After these fractions had been boiled, the inhibitory effect was enhanced considerably. Since the overall procedure involves several dialysis operations, it appears from these preliminary observations that the cell soluble fraction contains a non-diffusible, thermostable inhibitor, the activity of which may represent that part of the inhibition which resists dialysis (Table 7). No further study of the non-diffusible inhibitor has been made, although reports by Phipps[20a] and Stonier and Yoneda[20b] of high molecular weight inhibitors of IAA oxidase in tobacco and Japanese Morning Glory, respectively, may have some bearing on the problem.

Oxalate may contribute to the inhibition which is lost on dialysis (Table 7), since this acid has been isolated from the cell soluble fraction as its potassium salt[21] and identified by its equivalence against permanganate and by carbon, hydrogen and potassium analysis. Furthermore, the isolated compound and potassium oxalate behaved identically in inhibiting crocin destruction catalysed by the particulate system.

Evidence for the Participation of the Particulate System in the Loss of Carotene Accompanying Damage of Sugar Beet Leaf Tissue

The primary aim of our investigation has been to elucidate the nature of thermolabile catalysts responsible for the rapid oxidation of carotenoids which occurs when green leaf tissue is damaged. Evidence both for the participation of the system under investigation and for the use of crocin as a model substrate was derived from an experiment in which the destruction of endogenous carotene was measured in samples of macerated leaf in the presence and absence of 8-hydroxyquinoline. This compound has little effect on the activity of the system extracted by Triton-X-100.[1] Loss of carotene was stimulated by nearly 100 per cent in the presence of 8-hydroxyquinoline at an effective concentration of between 0·75 and 1·0 mM (Table 9).

[20a] J. PHIPPS, *Compt Rend. Acad. Sci. Paris* **261**, 3864 (1965).
[20b] T. STONIER and Y. YONEDA, *Ann. N.Y. Acad. Sci.* **144**, 129 (1967).
[21] B. J. FINKLE and D. I. ARNON, *Physiol Plant.* 7, 614 (1954).

TABLE 9. DESTRUCTION OF ENDOGENOUS CAROTENE IN SUGAR BEET LEAF HOMOGENATES

Treatment*	Carotene content (μg/g fresh leaf)	Carotene destruction (μg/g fresh leaf in 20 min)	% Destruction
1	56·9	—	—
2	48·5	8·4	14·8
3	40·3	16·6	29·2

Treatments:

1. 1 g fresh leaf tissue was ground in acetone, and pigments were extracted with acetone and light petroleum, after the addition of a few crystals of hydroquinone (antioxidant). Carotene was separated by thin-layer chromatography on silica gel G with benzene as developing solvent ($R_f = 0{\cdot}85$). The orange band was scraped off, eluted with ether and after evaporation of the solvent *in vacuo*, dissolved in light petroleum (b.p. 60–80°). Carotene was estimated spectrophotometrically ($E^{1\%}_{1\,cm}$ at 450 nm = 2500).
2. 1 g fresh leaf tissue was ground with 3 ml phosphate-citrate buffer at pH 3·7, and the homogenate left in the dark for 20 min. Then, after addition of hydroquinone, carotene was extracted as in treatment 1.
3. As for treatment 2, except that buffer containing 1 mM 8-hydroxyquinoline was employed.

Concluding Comment

In reviewing knowledge of enzymic destruction of carotenoids, Blain[22] was able to state in 1963 that:

"It would perhaps be premature to draw a line between unsaturated fat oxidases on the one hand and enzymes destroying carotene without added linoleate on the other."

Kirsanova[23] reported the presence of oxidases for carotene in radish juice and potato, and Nishida and Yamamoto[24] claimed that peroxidase was largely responsible for carotene oxidation in sweet potato. As far as the authors are aware, however, the peroxidase-containing system described in the present paper represents the only example in plant tissue of a catalyst of deteriorative carotenoid oxidation for which a lipid requirement has been precluded.

EXPERIMENTAL

Materials

Leaves from greenhouse-grown sugar beet (Sharpe's Klein Wanzleben E) were harvested as required.

Stock solutions of crocin were prepared by methanol extraction of commercial saffron.[25] Triton-X-100 was a gift from Rohm and Haas Co. Linoleic acid was purchased from the Hormel Institute, IAA from Fluka. Cytochrome *c* (60–70 per cent), peroxidase and other reagents tested for their effects on the activity of the particulate system were also obtained commercially.

Preparation of Washed Residue and Derivative Fractions

Isolation of mitochondria[26] and preparation of extract and washed residue by treatment of the particles with Triton-X-100[1] have been described previously. Acetone treatment of the washed residue involved the cautious addition of 9 vol. of precooled acetone (−15°), care being taken that the temperature of the mixture

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[23] V. A. KIRSANOVA, *Biokhimiya* **3**, 191 (1938); *Chem. Abstr.* **33**, 8640 (1939).
[24] K. NISHIDA and Y. YAMAMOTO, *Mem. Fac. Agr., Kagoshima Univ.* **1**, 62 (1952); *Chem. Abstr.* **47**, 8926 (1953).
[25] J. FRIEND and A. M. MAYER, *Biochim. Biophys. Acta* **41**, 422 (1960).
[26] A. M. MAYER and J. FRIEND, *J. Exptl Botany* **11**, 141 (1960).

did not exceed 0°. The pellet obtained on subsequent centrifugation at −7° was dissolved in 5 mM pH 7·0 phosphate buffer containing 0·4 M sucrose to yield preparation $R_{ac}p$. Lipids in the aqueous acetone supernatant were transferred to light petroleum, which was removed by evaporation, and dissolved in ethanol to yield preparation $R_{ac}s$ (Table 2).

Suspensions of both mitochondria (M) and washed residue (R_w) have been used as a source of the particulate system described in this paper. The type of preparation employed in individual experiments is stated at the relevant point in text, tables and figures.

Enzyme Assays

The routine assay procedure for crocin-destroying activity is based on a previously described method.[25] The 2-ml reaction mixture contained phosphate-citrate buffer (pH 3·6, 17 mM with respect to citrate), 140 mμmoles crocin, enzyme preparation and other reagents where appropriate. Flasks were shaken in a water bath at 30° for 20 min, after which a 1 ml sample was withdrawn for analysis. In assaying components of the cell soluble fraction which stimulate crocin destruction catalysed by the washed residue, a unit of "crocin-destruction stimulant" has been defined as the amount required to stimulate crocin destruction by 100 per cent in the presence of that amount of washed residue preparation which, unstimulated, destroys between 14 and 16 mμmoles of crocin in 20 min under routine assay conditions.

Peroxidase was assayed by a modification of the method of Haskins,[27] in which the oxidation of guaiacol is followed by measuring the increase in extinction at 460 nm. 2·65 ml 0·02 M pH 5·8 phosphate buffer, 0·10 ml 0·18 M guaiacol, 0·20 ml 0·03 M H_2O_2 and 0 05 ml of appropriately diluted enzyme solution were added to a cuvette of 1 cm light path. The reaction was started by the addition of enzyme, and increase in absorptivity measured in a Unicam SP600 at 1 min intervals over a period of 5 min against a blank containing all reagents except peroxide, which was replaced by water. A slight lag was sometimes observed at the start of the reaction; reaction velocity was measured between the first and the third minute. Assays were conducted at room temperature (22° ± 1) and a unit of peroxidase was defined as the amount required to bring about a ΔE_{460} of 0·10/min under the conditions defined. The assay of peroxidase in fractions obtained by treatment of mitochondria with Triton-X-100, performed at an early stage in this work (Table 1), differs from the procedure described above in that 0·05 M phosphate-citrate buffer (pH 3 5) was employed. Lower pH and the presence of citrate both contribute to a decrease in measured rates of guaiacol oxidation under these conditions. To compare the activity of particulate peroxidase assayed at pH 3·5 with that of peroxidase preparations assayed in 0·02 M phosphate buffer (pH 5·8), units of activity recorded in Table 1 should be multiplied by a factor of 2·2.

Catalase activity was measured by employing a modification[16] of the rapid titrimetric method of Bonnichsen *et al.*[28] 18·5 ml 0·1 M pH 7 0 phosphate buffer and 0·5 ml preparation were preincubated for 10 min in an ice bath prior to the addition of 1·0 ml 0·1 M H_2O_2 at zero time. 2-ml samples were withdrawn at 1, 2 and 3 min and pipetted into 0·5 ml of 5 N H_2SO_4. Residual peroxide was titrated with 0 01 N $KMnO_4$ until a faint pink colour was retained over 30 sec. The zero time titre was obtained by titrating a 2 ml sample taken from a mixture of 19 ml buffer and 1·0 ml H_2O_2 solution. Determination of the pH-activity relationship for particulate catalase was performed in 0·1 M phosphate-citrate buffers over a pH range of 2·5–7·8.

The reaction mixture employed in the assay of indole-acetic acid oxidase activity of the washed residue contained 1 ml 0·1 M phosphate-citrate buffer (pH range 2·6–6·1), 572 mμmoles IAA, 0·5 ml preparation, 2,4-dichlorophenol where appropriate and water to a final volume of 2 ml. Flasks were shaken in a water bath at 30° for 1 hr, after which a sample was withdrawn for analysis. Incubations were performed in duplicate in diffuse light, and any non-enzymic destruction was accounted for by including control flasks containing boiled preparation under all experimental conditions. Residual IAA was measured with Salkowski Reagent: a 1 ml sample from the reaction flask was pipetted into 3 ml freshly prepared reagent to give a final volume of 4 ml containing 2 6 M $HClO_4$ and 5 mM $Fe(NO_3)_3$, concentrations recently recommended by Perley and Stowe.[29] Precipitated material was removed from the mixture by centrifugation and extinction at 535 nm was measured after 40 min. Performing the estimation with known quantities of IAA confirmed that the extinction-concentration relationship was linear over the range 0–286 mμmoles.

Crocin Destruction by Peroxidase and Enzymically-Generated Hydrogen Peroxide

The complete system contained phosphate-citrate buffer at pH 3 6 (17 mM with respect to citrate), 20 mμM peroxidase (Koch-Light, RZ 1·0), 70 μM crocin, 140 μM *p*-coumaric acid and a peroxide-generating system composed of 500 μM D-glucose and 15 μg glucose oxidase (Boehringer, 90 per cent) in a reaction mixture of 4 ml. Flasks were shaken in a water bath at 30°. 0·5 -ml samples were withdrawn at zero time and at 2, 5, 8 and 10 min and pipetted into 5·5 ml methanol; extinction at 440 nm was measured No difference in rate (*ca.* 20 mμmoles crocin/min) was observed when *p*-coumaric acid was omitted: no oxidation occurred in absence of peroxide-generating system and *p*-coumaric acid.

[27] F. HASKINS, *Plant Physiol.* **30**, 71 (1955).
[28] R. K. BONNICHSEN, B CHANCE and H. THEORELL, *Acta chem. scand.* **1**, 688 (1947).
[29] J. E. PERLEY and B. B. STOWE, *Physiol. Plant.* **19**, 683 (1966).

Other Methods

Protein was determined by the method of Lowry *et al.*[30] Incidental procedures are described in the appropriate tables and figures.

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[30] O. H. LOWRY, N. J ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).