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

CROCIN OXIDATION BY AN UNSATURATED FAT OXIDASE SYSTEM IN TRITON EXTRACTS OF MITOCHONDRIA

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Abstract—The crocin-destroying system released from mitochondrial preparations of sugar beet leaves by treatment with Triton-X-100 is unstable under aerobic conditions at temperatures above 0°. Treatment of the Triton extract with acetone precipitates a heat-labile fraction with 15–40 per cent of the activity of the original extract. The supernatant is inactive alone, but increases the activity of the precipitate to 60–75 per cent. The supernatant factor is heat-stable and is effectively replaced by linoleic acid; varying the degree of peroxidation of added acid does not significantly influence the extent of stimulation. Cytochrome *c*, and to a lesser extent, peroxidase, will substitute for the acetone precipitate in catalysing crocin destruction in the presence of supernatant, but lipoxygenase is ineffective. The activity of the extract is inhibited by anerobic conditions, a variety of antioxidants, basic redox dyes, amino acids, cyanide and azide. Linolenic acid stimulates the activity of the extract more effectively than linoleic acid, and the effect of oleic acid appears to be concentration-dependent. A comparative study of the pH-activity relationships for the oxidation of methyl linoleate and crocin by Triton extract, cytochrome *c* and lipoxygenase has been made. The rates of oxidation of methyl linoleate and crocin by the extract are proportional to the square root of the concentration of extract. The collective data are considered to provide evidence for the presence of a haemoprotein-type unsaturated fat oxidase/lipid system in the Triton extract through which crocin oxidation is mediated.

INTRODUCTION

IN THE first paper of this series[1] it was shown that after treatment of sugar beet leaf mitochondria with the non-ionic detergent Triton-X-100, an extract and a residue were obtained each of which was active in catalysing the oxidation of crocin, the digentiobiose ester of the acidic carotenoid crocetin. In this present paper more detailed information is presented concerning the nature of the crocin-oxidizing system in the Triton extract of the mitochondria which appears to be an unsaturated fatty acid oxidizing system. A preliminary report of these findings has already been published.[2]

RESULTS AND DISCUSSION

Fractionation of the Crocin-destroying Activity in the Extract

Attempts to purify the heat-labile compounds responsible for crocin-destroying activity in the extract were made by ammonium sulphate fractionation, and by alteration of pH and exposure to increased temperature. Alteration of pH and temperature removed both active and inactive protein from the solution and gave preparations with reduced specific activity. Although complete recovery of activity was obtained when the starting solution was made 60 per cent saturated with ammonium sulphate, no increase in specific activity was found.

[1] J. FRIEND and J. W. DICKS, *Phytochem.* **5**, 205 (1966).
[2] J. W. DICKS and J. FRIEND, *Biochem. J.* **99**, 38P (1966).

Although the extract was stable for at least a month when stored frozen at −20°, a decrease in crocin-destroying activity was observed when the extract was either stored or manipulated at 2°; the half-life was about 7 hr. The rate of decay of the extract was slowed down by evacuating the vessel containing the extract (Fig. 1) but was unaffected by the addition of sodium thioglycollate, aminoethyl thiouronium bromide hydrobromide (AET) or polyvinyl pyrrolidone to the extract, or inclusion of ethylene diamine tetraacetate during the extraction procedure. An explanation for the loss of activity on storage is offered later.

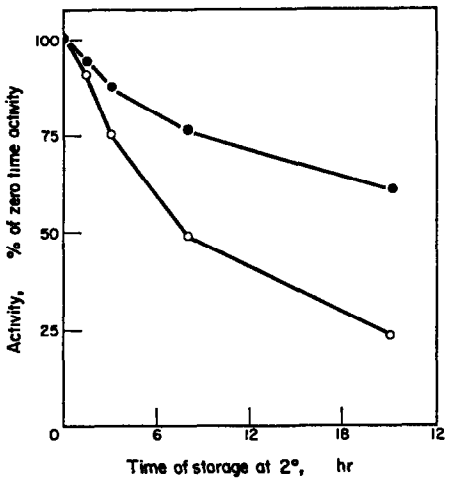

FIG. 1. THE EFFECT OF ANAEROBIC CONDITIONS ON THE RATE OF DECAY OF CROCIN-DESTROYING ACTIVITY IN THE TRITON EXTRACT.

Open circles: stored in air; closed circles: stored anaerobically.

Treatment of the extract with 2 vol. of cold acetone gave a precipitate (E_{Acp}) which had between 15 and 40 per cent of the activity of the original extract; the activity varied between different Triton extracts. The supernatant (E_{Acs}) had very little activity after the acetone had been removed. However, when the precipitate and the supernatant were combined about 60–70 per cent of the activity of the original extract was obtained; this was considerably more than the numerical sum of the two fractions separately (Table 1).

The effect of boiling the precipitate and the supernatant separately on the combined activities was measured (Table 1). The resuspended precipitate is inactivated by boiling.

TABLE 1. THE EFFECT OF ACETONE TREATMENT ON CROCIN DESTRUCTION BY THE TRITON EXTRACT (E_t)

Addition	Crocin destruction (% of E_t)
Triton extract (E_t)	100
Precipitate from E_t after acetone treatment (E_{Acp})	30
Supernatant from E_t after acetone treatment (E_{Acs})	13
Sum of E_{Acp} and E_{Acs}	43
$E_{Acp}+E_{Acs}$	62
E_{Acp} + boiled E_{Acs}	64
Boiled $E_{Acp}+E_{Acs}$	11

Since boiled precipitate and unboiled supernatant show only the activity of the supernatant, but the supernatant is unaffected by boiling, it was concluded that both the heat-labile factor in the residue and the heat-stable factor in the supernatant were required for full activity. Furthermore the heat-stable factor in the supernatant will not diffuse through dialysis membrane and it cannot be replaced by Triton-X-100.

Nature of the Heat-stable Factor in the Supernatant and Heat-labile Factor in the Residue

In an attempt to determine the nature of the heat-stable factor in the supernatant after acetone treatment, the addition of various compounds to the resuspended residue was examined.

TABLE 2. REPLACEMENT OF THE HEAT-STABLE FACTOR IN THE SUPERNATANT BY LINOLEIC ACID

Addition*	Crocin destruction (% of E_t)	
Triton extract (E_t)	100	100
Precipitate (E_{Acp})	15·5	37
Supernatant (E_{Acs})	3·2	4
$E_{Acp}+E_{Acs}$	62·3	73
Linoleic acid†	0	—
E_{Acp} + linoleic acid†	114	136

* See Table 1.

† Linoleic acid (containing 1% hydroperoxides) was at a final concentration of $3·2 \times 10^{-4}$ M.

TABLE 3. THE EFFECT OF ADDITION OF UNSATURATED FAT OXIDASES ON THE CROCIN-DESTROYING ACTIVITY OF THE HEAT-STABLE FACTOR IN THE SUPERNATANT

Additions to supernatant (E_{Acs})	Crocin destruction (ΔE_{440} nm in 20 min × 10^3)
None	5
0·1 mg Lipoxygenase	5
0·1 mg Peroxidase	35
0·1 mg Cytochrome *c*	78

Linoleic acid, containing about 1% hydroperoxide was found to stimulate crocin oxidation by the acetone precipitate (Table 2). Since it has been known for many years that carotenoids are readily oxidized in the presence of unsaturated fatty acids,[3] this merely indicates that the acetone precipitate has unsaturated fat oxidase activity. However, the effect of incubating known catalysts of unsaturated fat oxidation, in the presence of the acetone supernatant, with crocin was examined and it was found that, although lipoxygenase had no effect, both peroxidase and cytochrome *c* catalysed the oxidation of crocin in the presence of the acetone supernatant (Table 3). From the results of these experiments it was tentatively

[3] S. BERGSTROM and R. T. HOLMAN, *Advan. Enzymol.* **8**, 425 (1948).

concluded that the acetone precipitate contains an enzyme or other heat-labile catalyst which will catalyse crocin oxidation in the presence of an unsaturated lipid in the acetone supernatant.

This hypothesis was further supported by the finding that crocin oxidation by the extract was inhibited by anaerobic conditions, by the lipid antioxidants nordihydroguaiaretic acid, butylated hydroxyanisole and butylated hydroxytoluene, and by ascorbic acid and quinol (Table 4).

TABLE 4. THE EFFECT OF ANAEROBIC CONDITIONS AND SOME ANTIOXIDANTS ON CROCIN-DESTROYING ACTIVITY OF THE TRITON EXTRACT (E_t)

Inhibitor	% Inhibition
Anaerobic conditions	100
Nordihydroguaiaretic acid (NDGA)	100
Ascorbic acid	94
Butylated hydroxyanisole (BHA)	87
Butylated hydroxytoluene (BHT)	66
Quinol	60

The inhibition by the lipid antioxidants supports further a role of unsaturated lipid in crocin oxidation; the inhibition by anaerobic conditions is taken to imply that an actual oxidation of lipid is required to be in progress for crocin to be destroyed rather than that crocin destruction is dependent upon a supply of preformed hydroperoxide. Furthermore, an increase in the level of hydroperoxide in linoleic acid added to the acetone precipitate gave no parallel

TABLE 5. THE EFFECT OF VARYING HYDROPEROXIDE LEVELS AT A FIXED CONCENTRATION OF LINOLEIC ACID ON THE CROCIN-DESTROYING ACTIVITY OF THE HEAT-LABILE FACTOR PRECIPITATED BY ACETONE (E_{Acp})

Experiment 1	
% Hydroperoxide in 1×10^{-4} M linoleic acid	Crocin destruction (ΔE_{440} nm in 4 min $\times 10^3$)
0 (E_{Acp} alone)	75
0·2	124
1·1	124
2·0	135
6·1	138
9·5	130
13·2	133
Experiment 2	
% Hydroperoxide in $3{\cdot}2 \times 10^{-4}$ M linoleic acid	Crocin destruction (ΔE_{440} nm in 10 min $\times 10^3$)
0 (E_{Acp} alone)	70
0·2	260
0·6	280
1·0	295

increase in the amount of crocin destroyed (Table 5). If a "lipoperoxidase" enzyme of the type described by Blain and Styles[4] were involved then a proportionality between the amount of crocin destroyed and the amount of hydroperoxide added would have been expected.

There is an indication from the results in Table 3 that a haematin-type catalyst is more likely to be present in the extract than lipoxygenase and this hypothesis was further investigated by determining the effects of cyanide and azide, basic amino acids and redox dyes, and of adding fatty acids of different levels of unsaturation.

Crocin destruction by the extract is inhibited by cyanide and azide and by all the redox dyes and amino acids tested (Table 6). Cyanide and azide have no inhibitory action on lipoxygenase[3] and the dyes and amino acids which inhibit crocin oxidation are those which Tappel has shown to inhibit haematin-catalysed lipid oxidations,[5] because they form haemichromes which are less effective catalysts than the original haematins.

TABLE 6. THE EFFECT OF CYANIDE, AZIDE AND SOME BASIC NITROGEN-CONTAINING REDOX DYES AND BASIC AMINOACIDS ON THE CROCIN-DESTROYING ACTIVITY OF THE TRITON EXTRACT (E_t)

Inhibitor	Final concentration of inhibitor (mM)	% Inhibition
KCN	10·0	97·0
	1·0	87·0
	0·1	64·5
NaN_3	10·0	54·0
	1·0	15·5
	0·1	12·0
Malachite green	1·0	81·0
Methylene blue		67·0
Methyl violet		51·0
Tryptophane		16·0
Histidine		8·0

The effects of oleic, linoleic and linolenic acids on crocin oxidation by the extract were tested at two concentrations (Table 7). At the lower concentration, oleic acid had little effect but linolenic acid gave a much higher rate of oxidation than linoleic acid. However at the higher concentration oleic acid inhibited crocin oxidation although at this concentration stimulation by both linoleic and linolenic acids was intensified. If lipoxygenase were the catalyst, linoleic and linolenic acids would be oxidized at the same rate and oleic acid would act as a competitive inhibitor,[3] whereas in the case of a haematin catalyst the rate of oxidation should increase in the order oleic, linoleic, linolenic acids.[6] Since this latter situation was actually observed at the lower concentration of added fatty acid, it could be concluded that the extract contains a haematin type of catalyst. The finding which is not completely in accord with this conclusion is the inhibition by oleic acid at the higher concentration. However, it may well be that this oleic acid concentration is so much greater than that of the endogenous substrate that the overall rate of oxidation is regulated by the added acid. A similar situation would apply in the

[4] J. A. BLAIN and E. C. C. STYLES, *Production and Application of Preparations in Food Manufacture*, p. 150. Society for Chemistry and Industry, London (1961).
[5] A. L. TAPPEL, *Arch. Biochem. Biophys.* **50**, 473 (1954).
[6] A. L. TAPPEL, *Arch. Biochem. Biophys.* **44**, 378 (1953).

case of the linoleic and linolenic acids and this hypothesis would account for the greater differences in rate of crocin oxidation between all three fatty acids at the higher concentration.

TABLE 7. A COMPARISON OF THE EFFECTS OF OLEIC, LINOLEIC AND LINOLENIC ACIDS ON THE CROCIN-DESTROYING ACTIVITY OF THE TRITON EXTRACT (E_t)

Fatty acid	Final concentration of added acid (mM)	Crocin destruction	
		ΔE_{440} nm in 20 min × 10^3	% of control
None (E_t alone)	0	262	100
Oleic acid	0·4	270	103
	1·5	212	81
Linoleic acid	0·4	332	127
	1·5	377	144
Linolenic acid	0·4	390	149
	1·5	457	174

The fatty acids used in this experiment were free from measurable hydroperoxide contamination.

The assumption that crocin oxidation by the extract is coupled with the oxidation of an unsaturated lipid by a haematin-type catalyst can now be used to explain the loss of activity when the Triton extract is stored at 2°. The basis of the explanation is that primary substrate or catalyst or both are being destroyed in an endogenous reaction which can proceed at 2° since it is known that haemoproteins are active catalysts of lipid oxidation at 0°.[7] Storage in either the frozen or partly anaerobic state would be expected to inhibit such reactions and prevent the loss of activity. Addition of linoleic acid to stored extract still stimulates crocin destruction; the absolute amount of crocin destroyed declines but the percentage stimulation remains the same (Table 8). Since it would be expected that for a constant amount of catalyst the addition of a fixed amount of linoleic acid would catalyse the destruction of a standard amount of crocin, and this is not being observed, it is probable that catalyst is being destroyed during cold storage.

TABLE 8. THE EFFECT OF LINOLEIC ACID ON CROCIN DESTRUCTION BY THE TRITON EXTRACT (E_t) AT DIFFERENT STAGES DURING AEROBIC STORAGE OF THE EXTRACT

Additions	Crocin destruction (ΔE_{440} nm in 20 min × 10^3) at different times of storage of the extract		
	0 hr	7·5 hr	23·5 hr
Triton extract (E_t)	270	195	90
E_t and linoleic acid	405	295	135
% Stimulation by linoleic acid	50	52	50

Linoleic acid (containing 1% hydroperoxides), at a final concentration of $3{\cdot}2 \times 10^{-4}$ M was added at the times of assay reported, not to the extract for the period of storage.
Storage was carried out in air at 2°.

[7] A. L. TAPPEL, *J. Biol. Chem.* **217**, 721 (1955).

The Oxidation of Linoleate by the Triton Extract

The experiments so far reported gave results which supported the hypothesis that crocin oxidation by the Triton extract was a coupled oxidation in which an unsaturated lipid was being oxidized by a haematin-type catalyst. In order to confirm this hypothesis the nature of the catalyst was further investigated by examination of the oxidation of added methyl linoleate by the Triton extract. The oxidation of added linoleate was followed by spectrophotometric measurements of hydroperoxides as ferric thiocyanate by the method of Koch, Stern and Ferrari.[8]

In the initial experiments there was an induction period of about 3 min after the addition of extract before there was any detectable increase in hydroperoxide. However, if the buffer and substrate were pre-incubated with a small amount of Triton-X-100 (equivalent to the amount later to be added with the mitochondrial extract) then the induction period was abolished. In all subsequent experiments, therefore, the buffer and methyl linoleate were pre-incubated for 5 min with detergent in order to obtain reasonably standardized conditions.

After one sample of methyl linoleate had been incubated with the extract for 3 hr and a considerable increase in hydroperoxide found, a sample of the incubation mixture was diluted with 95% ethanol and a difference spectrum measured using as reference a similarly diluted sample from an incubation of methyl linoleate with boiled Triton extract. The difference spectrum showed a sharp peak at 237 nm which corresponded closely with a peak at 234 nm previously reported for conjugated diene hydroperoxide.[3] This indicated that when hydroperoxide increased conjugated diene was formed, and it was taken as evidence that the hydroperoxides formed were derived from oxidation of the added unsaturated lipid.

pH Activity Relationships for Oxidation of Unsaturated Fatty Acids by the Extract

The pH-activity relationship for the oxidation of added methyl linoleate by the Triton extract (Fig. 2(a)) shows a sharp maximum at pH 3·6; this compared reasonably well with the maximum for crocin destruction at pH 4·0 (Fig. 2(d)). However there was additional lipid oxidation at pH 8·0 but almost no crocin oxidation.

The pH-activity relationship for the oxidation of methyl linoleate catalysed by cytochrome *c* was measured and compared with the oxidation of crocin by cytochrome *c* and linoleic acid (Fig. 2(b) and (e)). Once again a good correspondence in a low pH optimum was found and the optima also corresponded with those previously found for crocin and linoleate oxidation by the Triton extract. However, even with cytochrome *c* as catalyst, some lipid oxidation was found at pH values where little crocin destruction could be detected and in addition the time course of peroxide formation observed with mixtures of methyl linoleate and cytochrome *c* appeared to vary with pH. It will be noticed that induction periods occur in the middle range of pH values, that there are maxima at pH 4 and pH 8 and that the pH 4 maximum becomes more prominent after longer incubation times.

Despite the differences between the pH optima for linoleate oxidation and crocin oxidation, the most striking feature is the close correspondence between the pH activity curve for crocin oxidation by the Triton extract of mitochondria and by cytochrome *c* plus linoleic acid. They are quite different from curves for lipoxygenase, linoleic acid and crocin where no detectable crocin destruction can be measured at pH 4 (Fig. 2(f)). The oxidation of methyl linoleate by lipoxygenase is also insignificant at low pH (Fig. 2(c)).

[8] R. B. Koch, B. Stern and C. G. Ferrari, *Arch. Biochem. Biophys.* **78**, 165 (1958).

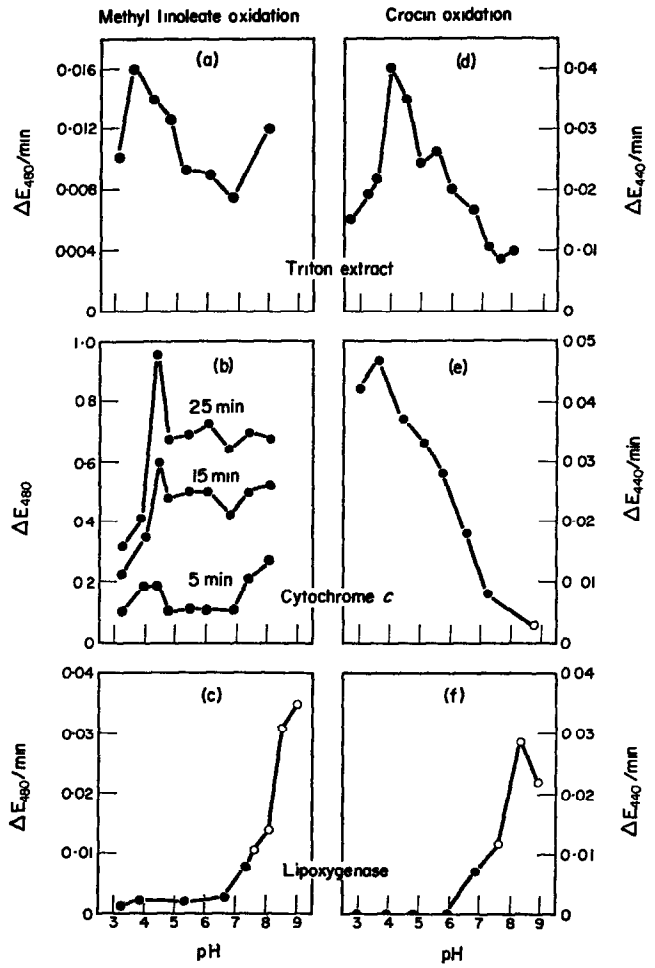

FIG. 2. pH-ACTIVITY RELATIONSHIPS FOR OXIDATION OF METHYL LINOLEATE AND CROCIN CATALYSED BY TRITON EXTRACT, CYTOCHROME *c* AND LIPOXYGENASE.

Catalyst concentrations: (a) and (d), 0·1 and 0·5 mg protein/ml respectively; (b) and (e), 1×10^{-5} M and 5×10^{-6} M cytochrome *c* respectively; (c) and (f), 20 μg/ml lipoxygenase in each case. Lipid substrate concentrations: (a,) (b) and (c), methyl linoleate at $1{\cdot}7 \times 10^{-3}$ M; (e) and (f), linoleic acid (containing 1% hydroperoxides) at $3{\cdot}2 \times 10^{-4}$ M. Open circles: borate buffers; closed circles: phosphate–citrate buffers.

The Relationship Between the Rate of Linoleate and Crocin Oxidation and the Concentration of the Extract

It was found when crocin destruction was measured during a small time interval (2 min) that the relationship between crocin destruction and the amount of extract was non-linear; also, with very small volumes of extract no crocin destruction could be measured. For methyl linoleate oxidation there was again a non-linear relationship between the oxidation and the amount of extract (Fig. 3(a)).

The two sets of data were re-plotted as reaction velocity against the square root of the volume of extract (Fig. 3(b)). In both bases, it will be seen that a linear relationship is now found and a close relationship between lipid and crocin oxidation by the extract demonstrated

once more. A linear relationship between the rate of reaction and the square root of the catalyst concentration is a characteristic feature of a free radical reaction and is found for lipid oxidations catalysed by haemoproteins but not by lipoxygenase.[9]

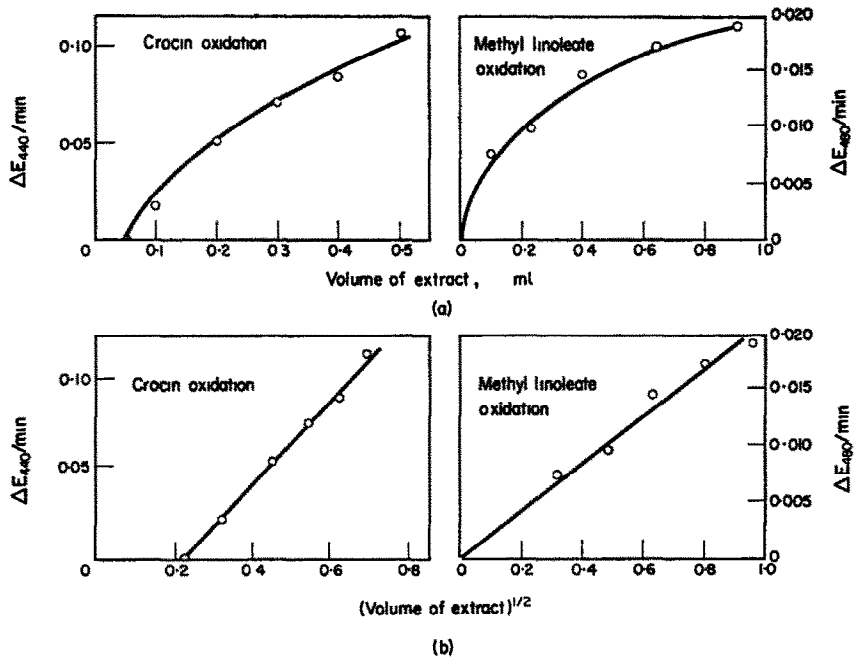

FIG. 3. THE RELATIONSHIP BETWEEN REACTION VELOCITY AND CATALYST CONCENTRATION FOR OXIDATION OF METHYL LINOLEATE AND CROCIN BY THE TRITON EXTRACT.

Methyl linoleate was added at a final concentration of $1{\cdot}7 \times 10^{-3}$ M.

It therefore seems reasonable to conclude from the weight of evidence that both crocin and lipid oxidations are catalysed by a haemoprotein-type catalyst in the Triton extract.

EXPERIMENTAL

Materials

Leaves from greenhouse-grown sugar beet (Sharpe's Klein Wanzleben E) were harvested as required, although on some occasions they were stored in polythene bags in a refrigerator for about 3 weeks without deterioration.

The preparation of stock solutions of crocin from commercial saffron has been described previously.[10] Highly purified fatty acids and their methyl esters were obtained from the Hormel Institute. Triton-X-100 was a gift from the Rohm and Haas Co. Lipoxygenase (lipoxidase) and peroxidase were obtained from L. Light and Co. and cytochrome *c* (60–70 per cent) from B.D.H. Ltd.

Methods

Treatment of mitochondria, isolated by the method of Mayer and Friend[11] with Triton-X-100 was described in the first paper of this series.[1]

Prior to acetone treatment of the Triton extract, the molarity of the solution was increased to 0·055 M by the addition of one-ninth volumes of cold 0·5 M NaCl. 2 vol. of pre-cooled acetone (−15°) were added cautiously, care being taken that the temperature of the mixture never exceeded 0°. After stirring for 5 min, the

[9] A. L. TAPPEL, *Autoxidation and Antioxidants* (Edited by W. O. LUNDBERG), Vol. 1, p. 343. Interscience, New York (1961).
[10] J. FRIEND and A. M. MAYER, *Biochim. Biophys. Acta* **41**, 422 (1960).
[11] A. M. MAYER and J. FRIEND, *J. Exptl Botany* **11**, 141 (1960).

precipitate which formed was removed by centrifugation at 3000 *g* for 10 min at −7°, and resuspended in a solution containing 1% Triton-X-100 and 0·005 M phosphate buffer, pH 7·0, to provide the preparation designated E_{Acp}. Some loss in activity of this preparation accompanied its storage in the frozen state. Acetone was removed from the supernatant by evacuation in a rotary evaporator at room temperature in dim light. The acetone-free solution (E_{Acs}) was stored in a refrigerator under N_2.

The routine assay procedure for crocin-destroying activity is based on that described by Friend and Mayer.[10] The reaction mixture contained 1·0 ml 0·1 M phosphate–citrate buffer (pH 4·1) and crocin, preparation (or other enzyme solution), and other reagent solutions where appropriate to a final volume of 2 ml. Fatty acids were added as a small volume of ethanolic solution, so that the final ethanol content did not exceed 5 per cent; controls with ethanol were run to take into account the possible inhibitory effect of this solvent. Solutions of linoleic acid containing different levels of hydroperoxide (used in the experiments recorded in Table 5) were made by mixing different proportions of reasonably peroxide-free acid with a sample which had been stirred in the presence of air under u.v. irradiation overnight, and which contained 13% hydroperoxide. A crocin concentration of 140 μg in 2 ml was found to be more convenient than the 70 μg used previously.[1,10] Where the measurement of initial reaction velocity was important (Figs. 2 and 3), an incubation time of 2 min was employed. Except where stated otherwise, incubations were of 20 min duration.

The assay for unsaturated fat oxidase activity is a modification of the method employed by Koch, Stern and Ferrari,[8] in which the production of ferric thiocyanate by reaction between thiocyanate, ferrous iron and fat hydroperoxides in samples of the reaction mixture is estimated spectrophotometrically. The reaction mixture consisted of 0·5 ml methyl linoleate (50 mg/ml in 40% acetone and 60% of 95% ethanol by volume), 1·0 ml 1% Triton-X-100 in water, 2·0 ml of Triton extract (or 1 ml of an appropriately diluted solution of other unsaturated fat oxidases and 0·02 M phosphate–citrate (pH 3·8) or borate buffer to a final volume of 50 ml. Substrate, detergent and buffer were pre-incubated for 5 min before the addition of enzyme. An incubation temperature of 30° was employed. 1-ml samples were withdrawn periodically and pipetted into 10 ml 95% ethanol, to which was added 0·2 ml of conc. HCl, followed by 0·025 ml of 5% ferrous ammonium sulphate in 3% HCl. 30 sec after the latter addition, 1 ml of ammonium thiocyanate was added and the ferric salt was determined at 3·5 min in a Unicam SP500 at 480 nm against a blank consisting of a similarly treated sample from a flask containing boiled preparation. The initial peroxide level was determined by taking a sample at zero time.

Protein was estimated by the method of Lowry *et al.*[12]

Acknowledgement—One of us (J. W. D.) was the holder of a studentship awarded by D.S.I.R. (now S.R.C.).

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