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

CROCIN OXIDATION BY MITOCHONDRIA

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Abstract—Mitochondrial preparations from sugar beet leaves are shown to catalyse the aerobic oxidation of the carotenoid glycoside, crocin. This oxidation has maximal activity at pH 3·6 and is different from the oxidation catalysed by chloroplasts which has an optimum near pH 7·5. The oxidation is partially inhibited by a variety of compounds including KCN and is stimulated by 8-hydroxyquinoline. A lipoperoxidase system may be involved but only as a small fraction of the overall activity of the mitochondria. After treatment of the mitochondrial pellet with Triton-X-100 an extract and a residue are obtained, each of which will separately catalyse crocin oxidation. Evidence is presented that this treatment has separated two independent crocin oxidizing systems in the mitochondria.

INTRODUCTION

THE BULK of the destruction of carotenoids which occurs when detached leaves are damaged is presumed to be caused by an enzymic oxidation.[1] During investigations into the enzyme-catalysed destruction of endogenous β-carotene in leaf homogenates and chloroplasts of sugar beet, preliminary evidence was obtained for a carotene-destroying system in the chloroplast, and possibly a second system outside the chloroplast.[2] This evidence was confirmed using crocin, a water-soluble carotenoid glycoside, as added substrate.[3,4]

In this paper we report experiments which show that particulate preparations from sugar beet leaves, which contain mitochondria, actively catalyse crocin destruction. The properties of this system differ in several respects from those of the chloroplast system, particularly in regard to pH optimum and response to inhibitors. A preliminary report of this work has already appeared.[5]

RESULTS AND DISCUSSION

Demonstration of Crocin-destroying Activity Outside the Chloroplast

(*a*) *Mitochondria.* The preparation which sediments at 20,000 *g* is called "mitochondrial". It is green in colour but differs considerably in its protein/chlorophyll ratio from the chloroplast preparation (sedimented at 1000 *g*) and moreover we have shown that there is succinate dehydrogenase activity in the "mitochondrial" fraction which is absent from the chloroplast fraction.

[1] V. H. BOOTH, *J. Sci. Food Agric.* **11**, 8 (1960).
[2] J. FRIEND and T. O. M. NAKAYAMA, *Nature* **184**, 66 (1959).
[3] J. FRIEND and A. M. MAYER, *Biochim. et Biophys. Acta* **41**, 422 (1960).
[4] J. FRIEND, *Production and Application of Enzyme Preparations in Food Manufacture*, p. 160. Society for Chemistry and Industry, London (1961).
[5] J. FRIEND and J. W. DICKS, *Biochem. J.* **88**, 37P (1963).

In preliminary experiments comparison was made between the crocin-destroying activities of the resuspended mitochondria and resuspended chloroplasts which showed that at pH 7·3 the chloroplasts were more active than the mitochondria. However, when the activities were measured at pH 4·7, in the pH range reported to be optimal for destruction of endogenous carotenoids in green leaf tissue[1] it was found that the mitochondria were more active than the chloroplasts.[4] More careful examination of the pH range revealed that the mitochondria were most active at pH 3·6. There was a second peak of activity at pH 7·5, at which pH the

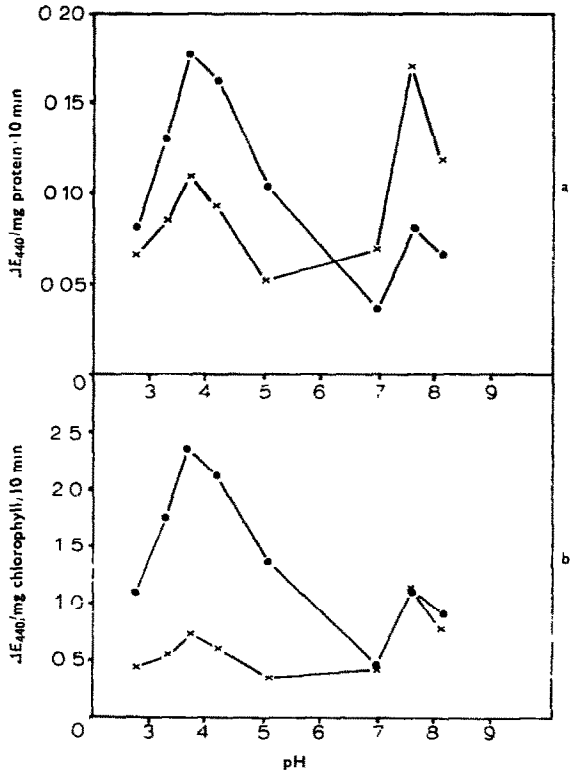

FIG. 1. EFFECT OF pH ON CROCIN DESTRUCTION BY SUSPENSIONS OF MITOCHONDRIA AND OF CHLOROPLASTS FROM SUGAR BEET LEAVES.

Upper graph: results plotted as activity per mg protein.
Lower graph: results plotted as activity per mg chlorophyll.
- - - mitochondrial suspension.
– ‹ – ‹ – chloroplast suspension.

chloroplasts were most active (Fig. 1a) and this was assumed to be caused by the contamination of the mitochondrial fraction with chloroplast fragments. The justification for this assumption is that if the crocin destruction of chloroplasts and mitochondria is compared on the basis of chlorophyll content (Fig. 1b) and not as activity per mg protein (Fig. 1a), it may be seen that the activity of the mitochondria at pH 3·6 is three times that of the chloroplasts.

The chloroplast fraction also shows a peak of activity at pH 3·6 which has about 60 per cent of the activity measured at pH 7·5. The suggestion is made that this low pH activity of the chloroplast fraction is caused by cross-contamination of the chloroplasts with mito-

chondria.[6,7] Some experimental evidence for this assumption is that chloroplasts purified by density-gradient centrifugation show less crocin destruction at pH 3·6 compared with the chloroplasts used in these experiments (Friend and Acton, unpublished).

(*b*) *Supernatant.* The supernatant remaining after centrifugal removal of the mitochondria showed little crocin-destroying activity at either end of the pH range. However, it was found that addition of supernatant to mitochondria markedly stimulated the destruction of crocin catalysed by the mitochondria. This feature is discussed more fully in a later section of the paper.

It was concluded from these results that there is an active crocin-destroying system outside the chloroplasts and that it is located in that fraction which contains the mitochondria.

Properties of the Crocin-destroying System in Mitochondria

(*a*) *pH optimum.* Measurements of mitochondrial activity between pH 3 and pH 4 confirmed that crocin-destroying activity was maximal at pH 3·6.

(*b*) *Effects of linoleate addition.* The coupled oxidation of carotenoids in the presence of

TABLE 1. THE EFFECT OF ADDED LINOLEATE ON CROCIN DESTRUCTION BY MITOCHONDRIA IN THE PRESENCE AND ABSENCE OF EDTA

	Crocin destruction (% of control activity) Linoleate (1×10^{-3} M)		
	None	Aged*	Fresh
Control	100	120	100
Anaerobic conditions	0	0	—
$+10^{-3}$ M EDTA	88·5	88·5	75
$+5 \times 10^{-5}$ M EDTA	87·4	92·3	—

* Linoleate hydroperoxide determined spectrophotometrically gave a final concentration of 1×10^{-6} M.

unsaturated fatty acids is well documented and forms the basis of several assay methods for oxidation of unsaturated lipids.[8] Since it has been found that one such oxidation, namely a "lipoperoxidase" reaction utilizing pre-formed linoleate hydroperoxide has an optimum near pH 3·8,[9] it was decided to examine the effect of linoleate addition on crocin destruction by mitochondria.

Addition of a freshly prepared suspension of sodium linoleate to mitochondria had no effect on crocin destruction whereas there was about 20 per cent stimulation upon addition of an "aged" linoleate solution which showed a hydroperoxide content of approximately 1 per cent (Table 1). The stimulation by the partially peroxidized linoleate was abolished by the addition of EDTA to the reaction mixtures; EDTA also inhibited crocin oxidation by mitochondria to which no additions had been made, or to which freshly prepared sodium linoleate had been added. The stimulatory effect of the linoleate hydroperoxide pointed to the probable involvement of a system utilizing partially peroxidized linoleate for a coupled

[6] W. O. JAMES and V. S. R. DAS, *New Phytol.* **56**, 325 (1957).
[7] R. M. LEECH and R. J. ELLIS, *Nature* **190**, 790 (1961).
[8] J. A. BLAIN, J. HAWTHORN and J. P. TODD, *J. Sci. Food Agric.* **4**, 580 (1953).
[9] J. A. BLAIN and E. C. C. STYLES, *Nature* **184**, 1141 (1959).

oxidation of crocin, possibly of the lipoperoxidase type. However, the likelihood of this sort of enzyme being concerned was lessened by the finding that there was complete inhibition of crocin destruction under anaerobic conditions, even when partially peroxidized linoleate was added (Table 1). Furthermore, pre-incubation of the mitochondria for two hours with added ascorbate, a procedure which was expected to increase the peroxidation of the endogenous lipid,[10] not only did not increase the amount of crocin destruction but actually caused a greater inhibition of crocin destruction than adding ascorbate just prior to the assay (Table 2; compare the result in Table 3).

TABLE 2. THE EFFECT OF PRE-INCUBATION OF MITOCHONDRIA IN BUFFER ON CROCIN DESTRUCTION

	Crocin destruction (% of control activity)
Control	100
Mitochondria* pre-incubated at 30° for 2 hr with buffer	91
Mitochondria* pre-incubated at 30° for 2 hr with ascorbic acid, sufficient to give a final concentration after addition of substrate of 1×10^{-3} M	24

* The mitochondria, buffer and ascorbic acid, when added, were shaken at 30° C for 2 hr; the flasks were then cooled, substrate added and then incubated as usual for assay of crocin destruction.

TABLE 3. THE EFFECT OF INHIBITORS AND OTHER TREATMENTS ON CROCIN DESTRUCTION BY MITOCHONDRIA

Compound added (all at a final concn. of 1×10^{-3} M)	Crocin destruction (% of control activity)
Control (no additions)	100
Quinol	18
Potassium cyanide	30
Ascorbic acid	42
Sodium diethyl dithiocarbamate (DIECA)	47
Sodium azide	59
o-Phenanthroline	70
Thiourea	78
Salicylaldoxime	129
8-hydroxyquinoline	161

The finding that these stimulations by added peroxidized linoleate were abolished by EDTA and that there was only between 10 and 15 per cent inhibition of mitochondrial oxidation of crocin at even higher concentrations of EDTA led us to assume that if coupled systems involving linoleate were concerned in crocin oxidation by mitochondria they could only represent between 10–15 per cent of the total mitochondrial activity.

(*c*) *Effects of inhibitors.* In order to obtain some clue as to the nature of the oxidizing enzyme system involved, the effects of a variety of inhibitors were examined (Table 3).

Hydroquinone (quinol) was found to be the most potent inhibitor of crocin oxidation. This compound is known to inhibit carotene oxidation during extraction from leaf tissue into

[10] A. OTTOLENGHI, F. BERNHEIM and K. M. WILBUR, *Arch. Biochem. Biophys.* **56**, 157 (1955).

organic solvents[11] and probably acts as an antioxidant. Ascorbic acid also probably acts as an antioxidant although it does not appear to be as effective as hydroquinone. The inhibitions by cyanide and azide suggest the possibility of involvement of a metallo-enzyme of the haematin type since *o*-phenanthroline and sodium diethyl-dithiocarbamate (DIECA) were not such effective inhibitors. The stimulatory effect of the metal chelating agents salicylaldoxime and 8-hydroxyquinoline should be noted; although these might appear puzzling at first sight, it should be borne in mind that 8-hydroxyquinoline stimulates oxalic acid oxidase, a flavoprotein oxidase, in *Bougainvillea* chloroplasts.[12] Since a flavoprotein oxidase would theoretically produce hydrogen peroxide which can oxidize crocin, and because it has been reported that oxalic acid oxidase is associated with a "particulate" fraction from sugar beet leaves[13] the effect of addition of oxalic acid and a variety of other substrates for flavoprotein oxidases was examined (Table 4). None of these compounds greatly affected crocin oxidation by the mitochondria. Even if a flavoprotein oxidase requiring one of these compounds as substrate were involved, the concentration of substrate may not have been a factor limiting crocin destruction.

TABLE 4. THE EFFECT OF THE ADDITION OF COMPOUNDS WHICH ARE POSSIBLE SUBSTRATES FOR FLAVOPROTEIN OXIDASES ON CROCIN DESTRUCTION BY MITOCHONDRIA

Compound*	Crocin destruction (% of control activity)
Control	100
Glycollic acid	110
L-Glycine	93
L-Glutamic acid	103
L-Lactic acid	110
Oxalic acid	82
D-Glucose	80
DL-Alanine (2×10^{-3} M)	89

* Concentration = 1×10^{-3} M unless otherwise indicated.

Extraction of Crocin-destroying Activity from the Mitochondria

After resuspension of mitochondria in 0·05 M phosphate buffer pH 7·0, or solutions of either 1% sodium deoxycholate or 1% digitonin in the same buffer, followed by centrifugation, only a small part of the crocin-destroying activity of the mitochondria remained in the supernatant.

However, treatment with Triton-X-100 was found to be an effective method of extracting activity. The mitochondrial pellet was macerated at 0° C with 1% Triton-X-100 in 0·005 M phosphate buffer pH 7·0 and left to stand for 30 min. Although suspensions of the mitochondria in Triton solution showed a slightly inhibited crocin destruction, after centrifuging the suspension for 30 min at 75,000 *g*, about 80 per cent of the activity remained in the supernatant fraction (Table 5). The arithmetical sum of the activities of the supernatant and residue show an increase in total activity of over 30 per cent compared with the suspension of mitochondria in Triton-X-100. Since a further increase in activity of the residue was found

[11] V. H. BOOTH, *Carotene: Its Determination in Biological Materials*. Heffer, Cambridge (1957).
[12] S. K. SRIVASTAVA and P. S. KRISHNAN, *Biochem. J.* **85**, 33 (1962).
[13] B. J. FINKLE and D. I. ARNON, *Physiol. Plant.* **7**, 614 (1954).

after resuspending it in buffer and re-sedimenting, presumably due to removal of occluded Triton-X-100, the total crocin-destroying activity which can be revealed after disruption in the detergent is almost double that of the original mitochondria (Table 5).

TABLE 5. THE DISTRIBUTION OF AND INCREASE IN CROCIN-DESTROYING ACTIVITY AFTER TREATMENT OF MITOCHONDRIA WITH TRITON-X-100

Treatment	Crocin destruction (% of 2)	Protein (% of 2)	Relative specific activity (crocin destruction/protein)
1. Mitochondria suspended in 0·4 M sucrose	100	97	1·03
2. Mitochondria suspended in 1% Triton-X-100	100	100	1·00
3. Supernatant after centrifugation of 2	107	57	1·88
4. Residue after centrifugation of 2 resuspended in 1% Triton-X-100	26	42	0·62
5. Residue after centrifugation of 2 washed and resuspended in buffer	73	33	2·21
Sum of 3+4	133	99	1·34
Sum of 3+5	180	90	2·00

Differences Between the Enzymic Activity in the Extract and that in the Residue

Since no additional activity could be extracted from the residue by a second treatment with Triton-X-100, it seemed probable that the activity in the extract and that in the residue represented two different enzyme systems each of which could catalyse the oxidation of crocin. Several features of the two systems were compared to see whether this hypothesis could be substantiated.

(*a*) *Effects of 8-hydroxyquinoline and cyanide.* It was decided to examine the effect of two compounds which had opposite effects on crocin oxidation by mitochondria, namely 8-hydroxyquinoline and potassium cyanide (Table 6). 8-Hydroxyquinoline has distinctly different effects on the extract and the washed residue, inhibiting the former slightly and stimulating the latter about 2·5-fold. Although there is a difference in the action of cyanide on the residue and on the extract, this reagent is not so useful for distinguishing two separate activities since its effect is obviously much more modified by the presence of Triton-X-100

TABLE 6. COMPARISON OF THE EFFECTS OF 8-HYDROXYQUINOLINE AND POTASSIUM CYANIDE ON THE SEPARATED FRACTIONS FROM MITOCHONDRIA

	8-hydroxyquinoline 1×10^{-3} M (% stimulation + or % inhibition −)	KCN 1×10^{-3} M (% inhibition)
1. Mitochondria suspended in 0·4 M sucrose	+76	−73
2. Mitochondria suspended in 1% Triton-X-100	+28	−22
3. Supernatant after centrifugation of suspension in (2)	−10	−35
4. Residue after centrifugation of suspension in (2) resuspended in 1% Triton-X-100	+112	−8
5. Residue from (4) washed and resuspended	+143	−57

than is the effect of 8-hydroxyquinoline. This feature may be seen when the first and second lines of Table 6 are compared.

The effect of 8-hydroxyquinoline was examined at lower concentrations (Table 7) and it can be seen that even at 50 μM it stimulated the activity of the washed residue. However, at the lowest concentration there is no inhibition of the activity of the supernatant.

TABLE 7. COMPARISON OF THE EFFECTS OF DIFFERENT CONCENTRATIONS OF 8-HYDROXYQUINOLINE ON THE CROCIN-DESTROYING ACTIVITY OF WASHED RESIDUE AND EXTRACT OBTAINED AFTER TREATMENT OF MITOCHONDRIA WITH TRITON-X-100

Concentration of 8-hydroxyquinoline (μM)	Crocin destruction (% of control)	
	Washed residue	Extract
0 (Control)	100	100
50	132	100
100	154	96·5
1000	230	90·5

(*b*) *Interaction with the soluble fraction of the homogenate.* The differences between the two fractions were further emphasized by a study of the effect of the soluble fraction of the leaf homogenate; we have already mentioned that addition of leaf supernatant to mitochondria stimulated the destruction of crocin. In a comparison of the addition of supernatant to mitochondria, Triton extract of mitochondria and washed residue, it was found that leaf supernatant had no effect on the Triton extract but that it stimulated the washed residue although not to quite the same extent that it stimulated the untreated mitochondria (Table 8).

TABLE 8. THE EFFECT OF ADDITION OF THE SUPERNATANT FROM SUGAR BEET LEAF HOMOGENATE ("CELL SOLUBLE FRACTION") ON CROCIN DESTRUCTION BY MITOCHONDRIA AND SUB-MITOCHONDRIAL FRACTIONS

	Addition of supernatant containing 0·43 mg protein (% stimulation)
Mitochondria suspended in 0·4 M sucrose	+260
Supernatant from Triton extract of mitochondria	0
Residue from Triton extract of mitochondria, washed and resuspended in buffer	+176

(*c*) *Time course of crocin destruction catalysed by the extracted enzyme system and by the mitochondrial residue.* When the amount of crocin destroyed at different time intervals using either the extract or residue as catalyst was compared, it was found that the two time curves were distinctly different. A linear relationship was maintained for at least 30 min with the residue, during which time 34% of the original crocin was destroyed. In contrast to this, the initial reaction velocity of crocin destruction catalysed by the Triton extract was maintained for only about 2 min, after which there was a decrease of the rate of reaction to almost zero after 15 min. Seventeen per cent of the crocin had been destroyed after two minutes, and it is possible, although not very probable, that this reduction in level of substrate may have been

a limiting factor for the overall activity shown by the extract and so have caused the observed reduction of the reaction rate. Even so, the rate of activity catalysed by the residue did not alter after 17 per cent of the crocin had been destroyed (15 min), and this difference in behaviour emphasizes still further the difference in the catalytic activity of the two enzyme fractions.

(*d*) *pH-Activity relationship.* An examination of the pH-activity relationship for crocin oxidation by the extract and by washed residue derived from the same mitochondrial pellet revealed a small but significant difference between these two fractions. In Fig. 2 it may be seen that the residue is similar to untreated particles in that maximum activity is found at pH 3·6, whereas in the extract an optimum is observed at pH 4·1 and there is also considerable activity at pH 5·2.

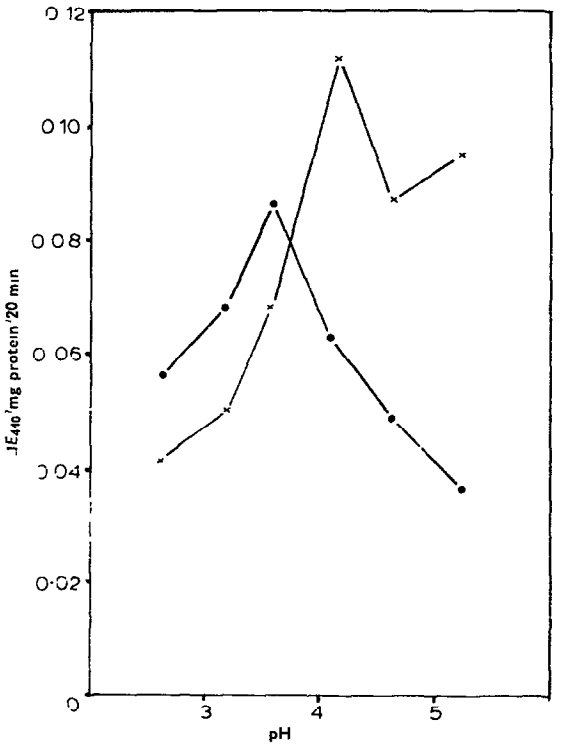

FIG. 2. EFFECT OF pH ON CROCIN DESTRUCTION BY WASHED RESIDUE AND EXTRACT OBTAINED AFTER TRITON TREATMENT OF CHLOROPLASTS.

·-·-· Washed residue.
-×-×- Extract

CONCLUSION

The oxidation of carotenoids which occurs in a subcellular fraction other than the chloroplasts has now been shown to be associated with the fraction containing mitochondria. Since the activity of the mitochondrial preparation is greatly stimulated by addition of the supernatant fraction from the leaf homogenate, it is reasonable to assume that this combined activity of mitochondria and supernatant will be responsible for carotenoid oxidation in damaged leaves.

The two sub-fractions of the mitochondria which were separated by treatment with Triton-X-100 have been shown to differ in respect of their response to selected inhibitors, in the effect of the soluble fraction from the leaf homogenate, in the rate at which they catalyse crocin destruction and in their pH-activity relationships. From these findings it is postulated that there are two separate enzyme systems in the mitochondria each of which will catalyse crocin destruction independently of the other when separated. It is interesting to note that, of the two fractions separated by treatment with Triton, it is the residue which has properties corresponding to those of the untreated mitochondria. Since the residue, after washing, has 73 per cent of the crocin-destroying activity of the mitochondria, it is possible that the activity shown by the mitochondria is mainly that of the residue. The activity of the extract may therefore be partially inhibited by the residue and only be displayed after separation of the extract and residue since the increase in total activity after Triton treatment is only revealed when the extract and residue are separated by centrifugation.

The properties of the extract and the residue have been examined in greater detail and will be described in the two following papers in the series.

EXPERIMENTAL

Materials

Sugar beet plants (Sharpe's Klein Wanzleben E) were grown in a greenhouse. Leaves were harvested when required; occasionally they were stored in a polythene bag in a refrigerator, since it was found they they could be stored for periods of 3–5 weeks without deterioration.

The method used for the isolation of the mitochondria was that of Mayer and Friend[14] except that the mitochondria were suspended in 0·4 M sucrose.

The method of extraction of the crocin from saffron and the preparation of stock solutions has been described previously.[3] The substrate solutions were made more concentrated so that a smaller volume could be used in the reaction mixtures. Triton-X-100 was a gift from the Rohm & Haas Co.

Methods

Mitochondrial suspension (usually 0·5 ml), 0·3 ml crocin solution, 0·2 ml water or solution of inhibitor and 0·1 M citrate–phosphate buffer to give a final volume of 2 ml were shaken in small conical flasks for 20 min at 30°. A 1 ml sample was withdrawn and analysed for residual crocin by the method of Friend and Mayer.[3] To compensate for possible non-enzymic oxidation of crocin, boiled mitochondria were incubated with crocin under identical conditions and the crocin estimated as described above. Chlorophyll was estimated by Arnon's method.[15] Protein was estimated by the method of Lowry *et al.*[16]

Triton-X-100 treatment of mitochondria was carried out by suspending the mitochondrial pellet in a solution containing 0·005 M phosphate buffer at pH 7·0 and 1% Triton-X-100. This suspension was left for 30 min at 0–2°, with occasional stirring. The volume of detergent solution used was the same as that of 0·4 M sucrose solution used for routine preparation of mitochondrial suspensions. The suspension was then centrifuged for 45 min at 75,000 *g* (30,000 rev/min in the 8 × 50 ml rotor of an M.S.E. Superspeed 50 centrifuge) and gave a

[14] A. M. Mayer and J. Friend, *J. Exptl. Bot.* **11**, 141 (1960).
[15] D. I. Arnon, *Plant Physiol.* **24**, 1 (1949).
[16] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).

clear, green supernatant (the extract) and a firm pellet (the residue). The latter was suspended in an equal volume of buffered Triton solution with the aid of an all-glass homogenizer: particulate material was removed by pouring the suspension through a double layer of gauze. However, when "washed residue" was required, the pellet from the high-speed centrifugation was resuspended in 0·005 M pH 7·0 phosphate buffer and recentrifuged. This pellet was finally resuspended in a solution containing 0·4 M sucrose and 0·005 M pH 7·0 phosphate buffer, since it was found that when sucrose was present the resuspended residue could be stored in the deep freeze (−20°) and thawed without any flocculation which otherwise occurred.

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