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AN IMPROVED PREPARATIVE METHOD FOR SPINACH CATALASE AND EVALUATION OF SOME OF ITS PROPERTIES

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SUMMARY

1. Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) has been purified from spinach (*Spinacea oleracea*) in 16% yield, by means of acetone fractionation, salting out by $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose and hydroxylapatite.

2. The purified enzyme had $s_{20,w} = 11.4$ S, $D_{20,w} = 3.75 \cdot 10^{-7}$ cm$^2 \cdot$sec^{-1}, $\bar{v} = 0.757$ cm$^3 \cdot$g^{-1}, hence the molecular weight was $3.0 \cdot 10^5$. The haem content was 0.93%. The enzyme aggregates irreversibly into 18 S, 23 S and higher species, when above 1–2 mg/ml concentration.

3. The activity was measured by means of a polarographic oxygen electrode. The rate constant for the purified enzyme was $k = 1.19 \cdot 10^6$ sec$^{-1} \cdot$M^{-1} (haem).

4. The principal spectral absorption maxima were 278 and 404 mμ ($E^{1\%}_{1\,cm}$ 14.8 and 14.9 respectively).

5. The enzyme crystallized from $(NH_4)_2SO_4$ in the monomolecular tube form.

6. Catalase was found to account for 0.56% of the acetone-soluble nitrogen of the spinach leaf. Less than 10% of the total catalase is associated with intact chloroplasts, and less than 1% with washed chloroplast lamellae. 90% was not associated with any subcellular particle.

INTRODUCTION

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) continues to present an enigma since its almost universal occurrence and high specific activity is not correlated with any agreed function. The plant enzyme, while believed to be located in chloroplasts (NEISH[1]), and hence potentially important in theories of normal or abnormal photosynthesis, has been little studied since GALSTON, BONNICHSEN AND ARNON[2] published a purification of spinach catalase and observed a low specific activity and haem content. This is virtually the only source of data concerning the plant enzyme, which, when compared with catalases from other sources (see NICHOLLS AND SCHONBAUM[3]), is insufficiently characterised.

The preparation of ferredoxin from spinach by the procedure of SAN PIETRO AND LANG[4], modified by SHIN, TAGAWA AND ARNON[5], yields a fraction rich in catalase activity; this offered the basis for an improved purification method for this enzyme.

In the present study the object was to obtain values for physical properties of the enzyme which could be compared with those known for animal and microbial catalases. Secondly, for the same comparative purpose, the quantity, activity and intracellular location of the enzyme in leaves was investigated.

METHODS AND MATERIALS

Assay of catalase activity

0.01 M phosphate buffer (pH 6.8) (equal molar quantities of KH_2PO_4 and Na_2HPO_4) was depleted in dissolved O_2, by bubbling N_2, and equilibrated in a water bath at 20°. 4.95-ml portions were transferred to the Chappell-Rank oxygen electrode (Rank Bros., Bottisham, Cambs., Great Britain) and 0.05 ml 0.20 M H_2O_2 added from a constriction pipette. The H_2O_2 solution was prepared from 20 vol. H_2O_2 A.R., which was standardised frequently by titration against standard $KMnO_4$. The H_2O_2 neither contained interfering substances, nor required neutralisation. The catalase sample, suitably diluted, was added from a 0.01-ml pipette, and the vessel closed. The concentration of O_2 was recorded against time on a strip-chart recorder, span 1 mV, speed 1 or 2 cm/min. The rate of O_2 evolution was obtained by drawing a tangent to the almost linear trace, sufficiently close to the start to make correction for the changed concentration of H_2O_2 negligible. Rates, after subtraction of the rate in the absence of catalase, were proportional to the quantity of catalase added, within 1% with rates up to 0.2 μmole O_2/min, and 5% up to 0.4 μmole O_2/min. It was convenient to define a unit of catalase activity as that quantity of enzyme which liberated 1 μmole O_2/min initially in this system. The method was compared with the rapid spectrophotometric assay of CHANCE AND MAEHLY[6], and the titration procedure of VON EULER AND JOSEPHSON[7], and the activities obtained all agreed within 2%.

Sedimentation and diffusion

The proteins were diluted so that $A^{1\,cm}_{278\,m\mu} = 1.0$, corresponding to 0.7 mg/ml. Sedimentation was performed at 42 040 rev./min and diffusion at 4770 rev./min in the Spinco model E ultracentrifuge using ultraviolet optics. The film (Kodak CF8) was analysed by means of the Beckman 'Analytrol' densitometer, and the curves corrected for the non-linearity of the response of that instrument to the concentration of the material in the ultracentrifuge cell. A temperature of 20.0° was maintained throughout, and observations at concentrations up to 2 mg/ml indicate that correction for concentration dependence of the sedimentation coefficient is negligible.

Polyacrylamide-gel electrophoresis

The equipment supplied by Shandon Scientific Co., Ltd., was used with tubes of 5 mm internal diameter. The gel concentration was 6% and the cross-linking 1.67%. The gels were polymerised with *N,N,N',N'*-tetramethyl-1:2-diaminoethane and ammonium persulphate, and were homogeneous. The buffers used for homogeneity tests on the purified enzyme were 0.01 M tricine–NaOH (pH 7.6) and 0.02 M tricine (pH 7.0). After 20 min pre-electrophoresis, the samples were applied to the upper surfaces of the gels (cathode) and a voltage of 200 V (2 to 6 mA/tube) applied for 30 min. The gels were then removed from the tubes, fixed and stained by amido black, 1% in methanol–

water–acetic acid (5:5:1, by vol.) for 10 min, then washed with the same solvent until the background was colourless.

Other measurements

Haematin was estimated by the method of HARTREE[8], assuming $\varepsilon_{mM} = 33$ for pyridine haemochromogen at the 558 mμ maximum. The mean of five determinations was taken. The dry weight of a given solution was measured by freeze-drying a 10-ml portion containing approx. 20 mg of protein, after dialysis for 3 days against five changes of twice-distilled water, during which time the spectral appearance of the material did not change. Duplicate samples from a calibrated 5-ml pipette were freeze-dried in squat, tared weighing bottles, in which the material was frozen in a thin film so as to avoid explosive evaporation. The protein was dried to constant weight over solid NaOH and P_2O_5 at 30°, being weighed rapidly with the lid in place. The partial specific volume of catalase was determined by pyknometry using approx. 2.4 ml of a solution containing 8.79 mg/ml catalase, dialysed against 0.01 M phosphate buffer (pH 6.8) for 24 h with stirring. Chlorophyll was estimated spectrophotometrically in 80% acetone using the equations of MACKINNEY[9]. Nitrogen was estimated by the micro-Kjeldahl method of CHIBNALL, REES AND WILLIAMS[10]. Ox-liver catalase (KAT 1) was obtained from Boehringer Corporation (London) Ltd.

Purification of catalase from spinach

Leaves were grown locally and harvested in batches of approx. 12 kg. They were washed and freed from the larger stalks and debris on the same day, and stored overnight at 4°. 1.5-kg portions were then homogenised in a 1-gal Waring blender at the middle speed for 20 sec with 1.08 l acetone at −15° and 500 ml 0.2 M Tris buffer containing 0.14 equiv. HCl/l (pH 7.7 at 20°). The homogenate was filtered through 68-μ aperture nylon bolting cloth (St. Martin's 21N, Henry Simon, Stockport) and centrifuged at 1000 × *g* for 20 min at 4°. The precipitate was discarded. To the combined supernatants were added 1.16 vol. of acetone chilled to −15°, and the mixture beaten and left to stand for 1 h in 18-l jars at 4°. All subsequent processes were carried out in the cold room at 4°. The clear yellow or pale green supernatant was then siphoned off and the pink-to-yellow sticky precipitate centrifuged down for 15 min at 1000 × *g*. The supernatant liquid was discarded and the solid suspended, using a Potter–Elvehjem homogenizer, in 250 ml of 0.02 M Tris chloride buffer as above containing 0.2 M NaCl, ice cold. This suspension was dialysed overnight against 2 changes of 15 l of the suspending medium with stirring. A bulky precipitate of insoluble material was then removed by centrifugation at 12 000 × *g*, and the dark solution remaining was passed through a column 3 cm × 15 cm, of DEAE-cellulose (Whatman DE 11) equilibrated with the suspending medium above. Ferredoxin was retained on the column and treated according to SAN PIETRO AND LANG[4]. 242 g solid $(NH_4)_2SO_4$, A.R. was added per l to the unretarded solution to bring it to 40% saturation. The precipitate that was formed was collected by centrifuging at 10 000 × *g* for 10 min and contained 50% of the total catalase extracted. The supernatant contained the flavoproteins and plastocyanin, and these were purified by the methods of SHIN, TAGAWA AND ARNON[5] and KATOH, SHIRATORI AND TAKAMIYA[11]. The precipitate above was dissolved rapidly in 150 ± 5 ml 0.02 M Tris buffer (pH 7.7), using the homogenizer to ensure very rapid dissolution and dilution. Often a colourless, inactive precipitate formed at this stage.

After dialysis to remove $(NH_4)_2SO_4$, the solution was refractionated by the successive additions of 94 g/l (to make it 17% saturated) and a further 89 g/l (31% saturation). The first precipitate contained variable amounts of an opalescent form of catalase that appeared to be an artifact produced during dissolution of the $(NH_4)_2SO_4$ precipitate. 80% of the catalase precipitated at the second addition. The supernatant remaining contained flavoproteins and was added to the appropriate fraction.

The catalase precipitated between 17 and 31% saturated $(NH_4)_2SO_4$ was dissolved in 150 ml (± 5 ml) of 0.02 M tricine adjusted to pH 7.4 with NaOH and dialysed to remove $(NH_4)_2SO_4$. The solution was then applied to a column 3 cm × 12 cm DEAE-cellulose equilibrated with 0.02 M tricine–NaOH buffer (pH 7.4). Tricine (GOOD[12]) was found to be essential as inadequate pH control was achieved with Tris. The catalase was bound to the column and, after washing, it was eluted with a gradient of the tricine buffer from 0.02 to 0.20 M, over 200 ml. A small variable quantity of catalase remained at the top of the column. Aggregates, when present (see below) preceded the main fraction.

The eluted catalase, with the absorbance ratio $A_{404\ m\mu}/A_{278\ m\mu}$ greater than 0.65, was carefully adjusted to pH 6.8 with 0.2 M acetate buffer (pH 5), and phosphate buffer (pH 6.8) was added to a final concentration of 0.01 M. The material was then applied to a column 2 cm × 8 cm of hydroxylapatite (LEVIN[13]) equilibrated with 0.01 M

TABLE I

PROGRESS OF THE PURIFICATION

The figures given are averaged over 5 preparations, scaled to 10 kg fresh leaf. At each stage the recoveries in the penultimate column, totalled and subtracted from 100%, indicate activity lost and unaccounted for. Bold type indicates the material used for the next stage.

Stage	*Material*	*Activity (units)*	*Specific activity*		*% recovery with respect to*	
			Units/mg	*Units/ml* $A_{278\ m\mu}^{1\ cm}$	*Previous stage*	*Original leaf*
—	**Fresh leaf** (10 kg)	1 050 000	0.105	—	—	100
I	**Solution** dialysed *vs.* Tris chloride	780 000	—	5–15	74.5	74.5
II	**Solution** after passage through DEAE-cellulose	770 000	—	30–40	99	73
III	$(NH_4)_2SO_4$ fractionation					
	ppt.65 –100% satn.	100	—	—	0	
	ppt. 40– 65% satn.	1 100	—	—	0–1	
	ppt. 0– 40% satn.	503 000	—	80	65	50
IV	$(NH_4)_2SO_4$ fractionation					
	ppt. 0–17% satn.	80 000	—	—	16	
	ppt. over 31% satn.	20 000	—	—	4	
	ppt. 17–31% satn.	390 000	—	150	78	38
V	DEAE-cellulose chromatography					
	$A_{404\ m\mu}/A_{278\ m\mu}$ ratio <0.65	33 000			8	
	$A_{404\ m\mu}/A_{278\ m\mu}$ **ratio >0.65**	246 000	—	410	63	23
VI	Hydroxylapatite chromatography					
	$A_{404\ m\mu}/A_{278\ m\mu}$ ratio <0.8	50 000	—	—	21	
	$0.95 > A_{401\ m\mu}/A_{278\ m\mu}$ **ratio >0.8**	25 000	—	600	10	2
	$A_{404\ m\mu}/A_{278\ m\mu}$ **ratio >0.95**	150 000	—	643	62	14
	Repetition of Stages V and VI					
	$A_{401\ m\mu}/A_{278\ m\mu}$ ratio 1.01	16 000	954	643		16

phosphate buffer (pH 6.8). The catalase was retained and after washing the column it was eluted with a gradient of phosphate buffer from 0.01 to 0.4 M over 150 ml. The bulk of the catalase appeared in the eluate at approx. 0.15 M phosphate. The central part of the fraction had absorbance ratio greater than 0.95. This fraction, and the remainder above the absorbance ratio = 0.80, were separately rechromatographed by one or both columns, when up to 16% of the starting activity was obtained with the absorbance ratio 0.95. The process is summarised, and the properties of successive stages of purification are recorded in Table I.

Preparation of intact chloroplasts

The density gradient method of LEECH[14] was used, except that the leaves were cut using an arrangement of razor-blades (Durham-Duplex Company, Ltd., Sheffield, England) rotating at 180 rev./min between stationary blades. All fittings were of stainless steel, and the apparatus was contained in an ice-bath. 200–300 g leaves were treated for 5 min in 800 ml 0.5 M sucrose, 0.04 M phosphate (pH 7.0) and the extract filtered through 25 μ aperture nylon bolting cloth (25TII Henry Simon, Stockport, England). Samples of this extract were then applied to the density gradients. The chloroplasts obtained were checked by light-microscopy. The yield was very small, but the chloroplasts were refractive, and showed balloon formation when osmotically shocked.

RESULTS

Purification

In the sequence given (Table I) the order of Stages V and VI may be reversed; the other conditions however are somewhat critical. The material of absorbance ratio greater than 0.95 gave a single band on polyacrylamide-gel electrophoresis at pH values 7.0 and 7.6, over a 10-fold range of protein concentration. Aggregates (see below) showed distinct bands, when present, behind the main one and the final stage is considered to yield catalase apparently homogeneous.

When ox-liver catalase was subjected to Stage VI, the absorbance ratio increased by 10%. A 20% decrease with some loss of activity was recorded when the enzyme was subjected to Stage V.

Properties

Spinach catalase was found to have the following properties: $s_{20,w} = 11.4$ S, $D_{20,w} = 3.75 \cdot 10^{-7}$ cm$^2 \cdot$sec^{-1}, $\bar{v} = 0.757$ cm $\cdot$ g^{-1}, from which the molecular weight is 300 000. The haematin content was 0.93 ± 03 (S.E.), so that the molecular weight is $(72\,000)n$; hence $n = 4$ with good agreement. Determinations of the molecular weight by the ARCHIBALD[15] technique were unreliable owing to the requirement that the concentration be kept low; values ranged from 280 000 to 320 000, with no progression in time.

The absorption spectrum is shown in Fig. 1. Values for the specific extinction coefficients $E^{1\%}_{1\,cm}$ are as follows: 248 mμ (min.) 8.51, 278 mμ (max.) 14.8, 313 mμ (min.) 3.41, 404 mμ (max.) 14.9, 478 mμ (min.) 1.91, 502 mμ (max.) 1.97, 533 mμ (shoulder) 1.71, 592 mμ (min.) 1.23, 620 mμ (max.) 1.38. Ox-liver catalase was found

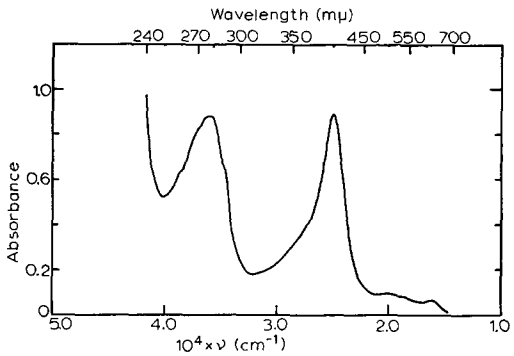

Fig. 1. Absorption spectrum of purified spinach catalase, 0.60 mg/ml in 0.1 M tricine–NaOH (pH 7.4), redrawn from a tracing on the Perkin–Elmer 'Uvicord' recording spectrophotometer. Positions and values of maxima and minima are given in the text.

to have maxima at 276 mμ and 405.5 mμ when compared directly with the spinach enzyme.

The specific activity was found to be 954 units/mg. The first-order velocity constant k in terms of haem was $1.19 \cdot 10^6$ sec$^{-1} \cdot$ M^{-1}. The 'Katalasefähigheit' (kal. f.) (VON EULER AND JOSEPHSON[7]) was 8290.

The purified catalase contained 14.4% nitrogen, whereas an acetone-dried spinach leaf powder prepared according to GALSTON, BONNICHSEN AND ARNON[2] contained 4.70% nitrogen. 1 g powder corresponded to 16 g fresh leaf which contained 110 units/g of catalase. Catalase therefore accounts for 0.56% of acetone-insoluble leaf nitrogen, and the activity in the leaf can be expressed as 37 400 units/g acetone-insoluble nitrogen.

The concentration of catalase in rat liver

A homogenate of rat liver, 0.065 g fresh weight/ml in 0.25 M sucrose, 1 mM EDTA–NaOH (pH 7.0) was assayed for catalase. The activity was found to be 74 units/ml, 1140 units/g fresh weight. Taking a figure of 3.2% (fresh weight) for the nitrogen content of rat liver (DE DUVE *et al.*[16]), the activity can be expressed as 35 600 units/g nitrogen.

Aggregation

On occasions when purified spinach catalase was left in solution at concentrations above 1 mg/ml, as for example when preparing for ultracentrifugal analysis by schlieren optics, additional components were observed with sedimentation coefficients of approximately 18 and 23 S. The density of the photographic image indicated that each of these components absorbed visible light, so that they can be regarded as catalase aggregates. They are eluted from columns of hydroxylapatite and DEAE-cellulose in advance of the 12-S material, but have not been obtained pure. The aggregates do not dissociate on dilution. There was a loss of some 20–30% of activity associated with aggregation when less than half of the 12-S fraction remained (by estimation of the areas under the schlieren curve). Hence it is probable that the 18-S species, at least, has catalase activity. Another catalase species was sometimes ob-

served which was precipitated by 17% saturated $(NH_4)_2SO_4$ at Stage IV. This material was opalescent. It appeared to be formed when precipitates containing catalase were dissolved slowly, or in a small volume. In all cases a serious drop in total activity resulted, and the material when free from the 12-S enzyme had approx. 10% of the specific activity on the basis of 404-mμ absorption. This species of catalase has not been purified from contaminating proteins.

Storage

Provided that the concentration of catalase is not raised above 1 mg/ml the purified material can be kept for at least 8 weeks at 4° in dilute phosphate or Tris buffers, pH 6.8–7.5, in the presence of thymol, without apparent change. Freezing and storage at −15° resulted in considerable formation of aggregates; this was reduced by rapid freezing in a bath of acetone–solid CO_2.

The catalase crystals

Approx. 18 mg catalase, $A_{404\ m\mu}/A_{278\ m\mu}$ ratio = 0.97, were dissolved in approx. 1 ml 0.1 M tricine–NaOH (pH 7.7) and saturated $(NH_4)_2SO_4$ solution added dropwise until a precipitate appeared. This was dissolved with about 0.02 ml of water, and the material left to stand at 4°, saturated $(NH_4)_2SO_4$ solution being added dropwise every 4 days for 2 weeks. When observations were resumed 5 weeks later, all the colour of catalase had precipitated, the precipitate showing 'silkiness' when agitated. When examined under the light microscope using phase-contrast, at extreme magnification, the precipitate had the appearance of a felt of fine needles, this being however a subjective description probably based on diffraction effects. Single drops of the suspension were then mixed with single drops of either potassium phosphotungstate, uranyl acetate or ammonium molybdate, each 1% in water, on grids coated with collodion and carbon. Excess liquid was drawn off with filter paper and the grids dried and examined under the Philips EM-100 electron microscope. All three negative-staining agents gave similar photographs: an example is reproduced in Fig. 2. The interpretation is the same as that given by KISELEV, SHPITZBERG AND VAINSHTEIN[17] for animal catalase crystals, *i.e.* that the crystals are monomolecular tubes. The tubes are apparently flattened on the grid.

The crystals were soluble in dilute buffers and their specific activity was not significantly different from the original preparation. After storage at 4° for 19 months, the fibrillar structure persisted but the molecular pattern was less prominent. When dissolved, the catalase had $A_{404\ m\mu}/A_{278\ m\mu}$ ratio = 0.84, activity 210 units/$A_{278\ m\mu}$ and a sedimentation coefficient of 11 S; no aggregates were observed.

Intracellular location

Leaves of *Spinacea oleracea*, *Beta vulgaris*, *Petroselinum crispum* and *Vicia faba* were thoroughly homogenised, with a pestle and mortar, in 0.05 M Tris chloride (pH 7.4), and assayed for catalase activity and chlorophyll content. See Table II. Intact chloroplasts from spinach, beet and Vicia leaves were assayed for catalase and chlorophyll. The chloroplasts were ruptured by homogenization in phosphate buffer (pH 7.0) using a Potter–Elvehjem homogenizer, and the lamellae obtained by centrifugation at 104 000 × *g* for 20 min. They were washed and assayed again. The results are shown in Table III.

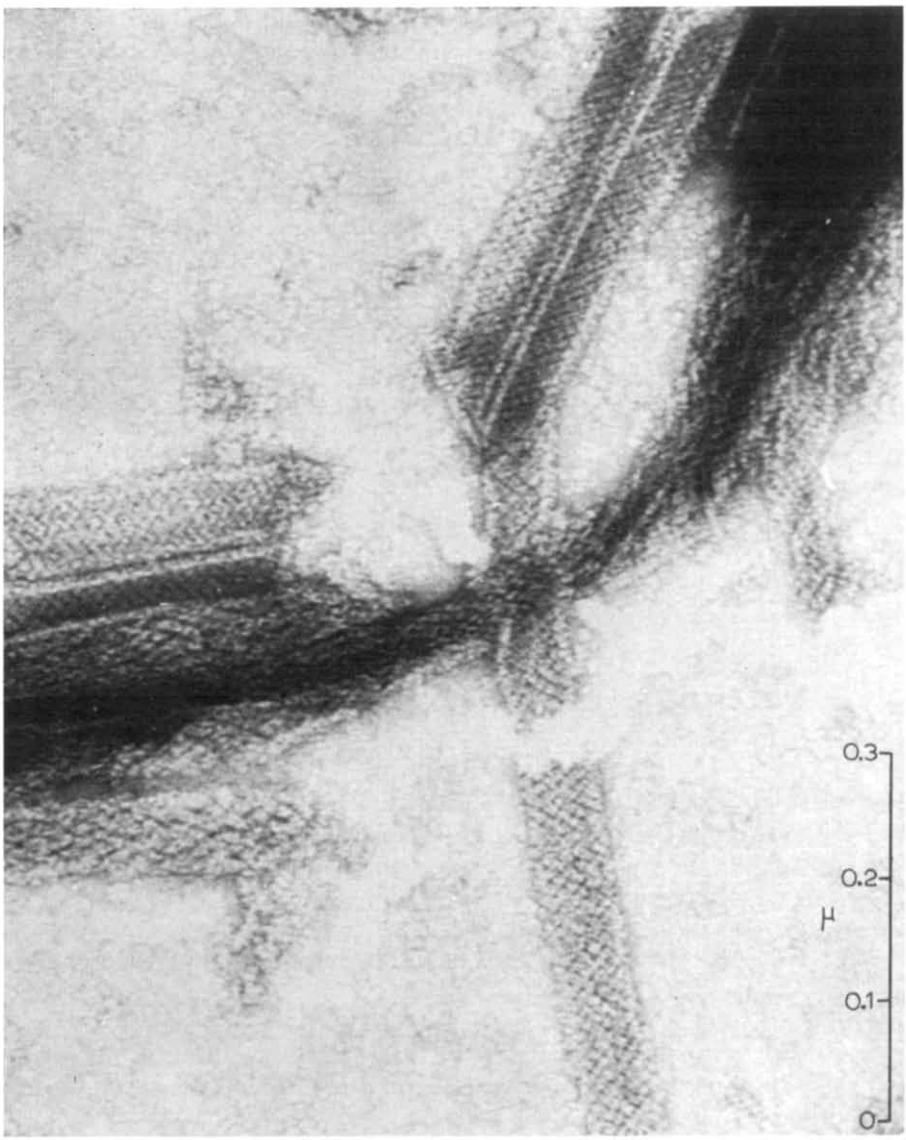

Fig. 2. Electron-micrograph of spinach catalase crystals, negatively stained with ammonium molybdate, to show the monomolecular tubular structure, and tendency of the tubes to bunch together.

A homogenate of spinach in 0.5 M sucrose, 0.05 M Tris (pH 7.4) after removal of chloroplasts, was assayed for catalase before and after centrifugation at 104 000 × *g* for 2 periods of 1 h each. The activity of the respective supernatants was 98 and 95% of the original. The soluble catalase cannot be associated with any subcellular particle, since all particles would have been sedimented to a greater or lesser extent in this experiment.

The density gradient technique ensures that the chloroplasts possess their

TABLE II

MEAN VALUES FOR CATALASE ACTIVITY AND CHLOROPHYLL CONTENT IN WHOLE LEAF HOMOGENATES OF 4 PLANT SPP.

The leaves were superficially dry and the midribs removed, except in the case of Spinacea (i) in which the leaves were as used for enzyme extraction, wet and with substantial rib and petiole material.

	Spinacea oleracea		*Beta vulgaris*	*Petrose-linum crispum*	*Vicia faba*
	(i)	*(ii)*			
Catalase (units/g)	110	228	227	420	193
Chlorophyll (mg/g)	0.66	1.3	0.90	1.74	1.78
Ratio (units/mg)	183	175	252	241	108

normal complement of stroma. LEECH[14] showed by electron microscopy that the limiting membrane is nearly always present, although this was not checked in the present study. The low ratios of catalase to chlorophyll in these chloroplasts (Table III: 4, 6 and 11% with respect to the whole leaves) are therefore a genuine indication that the bulk of the leaf catalase is external to the chloroplast. The second feature of the density gradient method is the virtually complete removal of the original cytoplasmic solution from the intact chloroplasts, so that the percentages above are minima.

TABLE III

CATALASE/CHLOROPHYLL RATIOS IN CHLOROPLASTS AND LAMELLAE

Data are expressed as units/mg.

	Spinacea oleracea	*Beta vulgaris*	*Vicia faba*
Original leaf	228	252	108
Chloroplasts	9	16	12
Lamellae	0.7	6	1.6

The catalase/chlorophyll ratios of the washed lamellae given in Table III are only 0.3, 2.4 and 1.5% of the ratios of the original leaves. It is known (*e.g.*, RIDLEY, THORNBER AND BAILEY[18]) that even after five aqueous extractions, stroma components are by no means completely removed from chloroplast lamellae, so that in the present case the catalase activity of the lamellae themselves must be lower than the figures given, possibly approaching zero.

DISCUSSION

The preparation of ferredoxin and other proteins is a routine operation of many laboratories in which photosynthesis is studied, so that the preparation of catalase may be made very economical. This method of preparation gives a good yield and presents less difficulty than previously[2]. It appears probable from the $(NH_4)_2SO_4$ limits

given by GALSTON, BONNICHSEN AND ARNON[2], the electrophoretic behaviour and the spectrum of their enzyme that it was an aggregated form. These are formed very readily during $(NH_4)_2SO_4$ fractionation unless the precautions described above are taken.

The spectral absorbance ratio of the final product is raised from 0.65 to 1.01. However the specific activity appears to be lower than that of GALSTON, BONNICHSEN AND ARNON[2] by a factor of 2.8, and the absorbance at 280 mμ that they quote is higher than that reported here by about the same factor. A single adjustment of the value of the dry weight of the earlier preparation would bring the two reports into harmony.

Spinach catalase now appears to be more similar to the enzyme from animal sources in molecular weight, number of haematin groups and spectrum. On the other hand, the specific activity is still lower, by a factor of 5–10 (see the review by NICHOLLS AND SCHONBAUM[3]). At the same time the comparatively great abundance of the spinach enzyme, 0.56% of acetone-insoluble leaf nitrogen, results in a net activity per unit weight of nitrogen comparable to that of rat liver.

Assuming that all catalases are phylogenetically homologous, the situation in spinach suggests that the enzyme may have been re-acquired since being genetically degraded in some ancestral species which had little or no requirement for it. The present form could differ from the original by substitution of, or addition to, the peptide chains; the higher molecular weight of the spinach enzyme suggests that the latter is more likely. Recent work on the preparation of sub-units of animal catalase (SUND, WEBER AND MÖLBERT[19]) might allow a direct comparison to be made between corresponding components of the two enzymes.

The observation by NEISH[1] that catalase was a chloroplast enzyme cannot be entirely supported, since in all the species examined the ratio of catalase to chlorophyll in intact chloroplasts was only 5–10% that of the whole leaves. Nevertheless, the catalase activity that remained in washed lamellae generated O_2 from 1 mM H_2O_2 at a greater rate than the HILL reaction did with 1 mM ferricyanide. Taking the figure of JAMES AND LEECH[20] for the ratio of cytochrome *f* to chlorophyll, and that of FORTI, BERTOLÈ AND ZANETTI[21] for the catalase activity of cytochrome *f*, the catalase activity of chloroplast lamellae is far less than that to be expected on the basis of their cytochrome-*f* content alone. Apart from the residual activity of washed lamellae, leaf catalase appears to be freely soluble, in contrast to its location in animal cells in light lysosomes or microbodies[22].

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