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# Purification and biosynthesis of cottonseed (*Gossypium hirsutum* L.) catalase

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As part of our research on peroxisome biogenesis, catalase was purified from cotyledons of dark-grown cotton (*Gossypium hirsutum* L.) seedlings and monospecific antibodies were raised in rabbits. Purified catalase appeared as three distinct electrophoretic forms in non-denaturing gels and as a single protein band (with a subunit $M_r$ of 57000) on silver-stained SDS/polyacrylamide gels. Western blots of crude extracts and isolated peroxisomes from cotton revealed one immunoreactive polypeptide with the same $M_r$ (57000) as the purified enzyme, indicating that catalase did not undergo any detectable change in $M_r$ during purification. Synthesis *in vitro*, directed by polyadenylated RNA isolated from either maturing seeds or cotyledons of dark-grown cotton seedlings, revealed a predominant immunoreactive translation product with a subunit $M_r$ of 57000 and an additional minor immunoreactive product with a subunit $M_r$ of 64000. Labelling studies *in vivo* revealed newly synthesized monomers of both the 64000- and 57000-$M_r$ proteins present in the cytosol and incorporation of both proteins into the peroxisome without proteolytic processing. Within the peroxisome, the 57000-$M_r$ catalase was found as an 11S tetramer; whereas the 64000-$M_r$ protein was found as a relatively long-lived 20S aggregate (native $M_r$ approx. 600000–800000). The results strongly indicate that the 64000-$M_r$ protein (catalase?) is not a precursor to the 57000-$M_r$ catalase and that cotton catalase is translated on cytosolic ribosomes without a cleavable transit or signal sequence.

## INTRODUCTION

Catalase (hydrogen-peroxide:hydrogen peroxidase oxidoreductase; EC 1.11.1.6) is a haemoprotein with an $M_r$ of 220000–240000, consisting of four similar subunits, and is a principal and characteristic enzyme of animal [1–4] and plant [5–8] peroxisomes. Catalase has been purified and characterized from various animal, fungal and bacterial sources [9] and from various plant sources, e.g., spinach (*Spinacia oleracea*) leaves [10]; lentil (*Lens culinaris*) leaves [11]; cucumber (*Cucumis sativus*) cotyledons [12]; sweet-potato (*Ipomoea batatas*) roots [13]; maize (*Zea mays*) scutella [14]; pumpkin (*Cucurbita* sp.) cotyledons [15–17]; and sunflower (*Helianthus annuus*) cotyledons [18].

As part of an ongoing project on peroxisome (glyoxysome) biogenesis and differentiation in cotton (*Gossypium hirsutum*) seeds, catalase from cotyledons of dark-grown cotton seedlings was purified for the purpose of studying the mature protein and generating monospecific antibodies in rabbits. Antibodies raised to purified cottonseed catalase were used to examine catalase synthesized *in vitro* and *in vivo*; the results have an important bearing on current concepts of glyoxysome biogenesis and the controversies related to the biogenesis of plant catalase.

## MATERIALS AND METHODS

### Chemicals

Acetone, ethanol, chloroform and ethylene glycol were purchased from J.T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Hydroxyapatite (Bio-Gel HT) was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. DEAE 23 SS Servacel was from Serva Fine Biochemicals, Heidelberg, Germany. L-[$^{35}$S]Methionine (1000–1300 Ci/mmol, 1 mCi/0.1 ml of 50 mM-Tricine, pH 7.4) was purchased from NEN–du Pont, Boston, MA, U.S.A. Guanidine thiocyanate was obtained from Fluka Chemical Corp., Hauppage, NY, U.S.A. CsCl was obtained from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A. All other materials were obtained as described previously [19,20].

### Buffers

Buffer pH values were determined at 25 °C. Buffers used in this study were composed as follows: KPB, $K_2HPO_4/KH_2PO_4$ (molarity and pH varies as stated below)/1 mM-EDTA/1 mM-PMSF/1 mM-benzamidine hydrochloride; PBS, 10 mM-$Na_2HPO_4/NaH_2PO_4$ (pH 7.2)/150 mM-NaCl; Buffer A, 1% (w/v) Triton X-100/150 mM-NaCl/50 mM-Tris/HCl/20 mM-methionine/1 mM-benzamidine hydrochloride/1 mM-iodoacetamide/3 mM-EDTA (pH 7.5)/1 mM-PMSF/leupeptin (25 $\mu$g/ml)/aprotinin (2 $\mu$g/ml); Buffer B, same as Buffer A, except with 0.1% (v/v) Triton X-100 and 0.02% (w/v) SDS.

### Plant material

Cotton [*Gossypium hirsutum* L. cv. Deltapine 62 (Delta and Pine Land Co., Lubbock, TX, U.S.A.)] seeds were germinated and grown under the conditions described previously [19]. Cucumber seeds (*Cucumis sativus* L. cv. Improved Long Green) and pumpkin (*Curcurbita* sp. cv.

Abbreviations used: DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide gel electrophoresis; poly(A)$^+$, polyadenylated; dpa, days post anthesis.

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Amakuri Nankin) seeds were kindly provided by Dr. W. Becker (Department of Botany, University of Wisconsin, Madison, WI 53706, U.S.A.), and Dr. J. Yamaguchi (Research Institute for Biochemical Regulation, School of Agriculture, Nagoya, Japan) respectively. Cotton plants were grown as described by Kunce *et al.* [21]. Embryos were removed from the ovules and cultured by the method of Choinski *et al.* [22] in nutrient medium containing 58 mM-sucrose and 3.8 $\mu$M-abscisic acid.

**Protein determination and catalase enzyme assay**

Protein and catalase activity were assayed as described previously [19].

**Gel electrophoresis**

Non-denaturing and SDS/PAGE were performed as described previously [19], except that for SDS/PAGE the reduced proteins were alkylated by using procedures described by Fujiki *et al.* [23]. For SDS/urea gels, samples were boiled for 15 min in 4% (w/v) SDS and 10% (v/v) mercaptoethanol, diluted with an equal volume of 4% SDS, 10% mercaptoethanol and 8 M-urea, and subjected to SDS/PAGE in the presence of 4 M-urea. Gels were stained for protein with silver by the method of Wray *et al.* [24].

For fluorography, Coomassie Blue-stained gels were impregnated with Fluoro-Hance (Research Products International Corp., Mount Prospect, IL, U.S.A.) according to the directions of the supplier, dried and exposed to preflashed X-Omat XAR-5 film.

**Catalase purification**

All procedures were performed at 4 °C unless specified otherwise. All buffers were de-aerated thoroughly before use. Cotyledons (450 pairs, 68 g fresh wt.) of cotton seedlings grown in the dark for 3 days were frozen in liquid $N_2$ and ground to a powder in cold mortars. Grinding was continued with 1800 ml of cold acetone (−20 °C). The suspension was centrifuged at 27000 *g* for 15 min. The resultant pellets were resuspended in 200 ml of 0.1 M-KPB, pH 7.5, and homogenized in a glass blender at high speed for 40 s. This homogenate was centrifuged at 27000 *g* for 60 min; pellets were discarded. The supernatant was fractionated with 0.8 vol of 95% (v/v) ethanol/chloroform (3:1, v/v) with 1 mM-PMSF as described by Schiefer *et al.* [11]. The upper aqueous layer was centrifuged at 23500 *g* for 30 min. The supernatant was added to DEAE-Servacel (12 g dry wt.) equilibrated previously in 0.1 M-KPB, pH 7.5. Immediately after the ion-exchanger was suspended thoroughly in the sample, it was centrifuged at 9880 *g* for 45 min. The supernatant was fractionated with 2 vol. of 95% ethanol with 1 mM-PMSF as described by Schiefer *et al.* [11]. Precipitated catalase was resuspended in approx. 50 ml of 0.1 M-KPB, pH 7.8, and dialysed for 16 h against 1800 ml of the same buffer. Thereafter, the enzyme solution was dialysed for 9 h against 0.37 vol. of 100%-saturated $(NH_4)_2SO_4$ (27% saturation at equilibrium), previously titrated to pH 7.8 with aq. $NH_3$. The precipitate was removed by centrifugation at 27000 *g* for 60 min, resuspended in approx. 10 ml of 10 mM-KPB, pH 7.8, and dialysed for 16 h against 1800 ml of the same buffer. After $(NH_4)_2SO_4$ fractionation, catalase was relatively inactive until desalted by dialysis.

The enzyme solution was applied to a hydroxyapatite column (1.4 cm × 12 cm) equilibrated previously in 10 mM-KPB, pH 7.8. The column was washed with 100 ml of the same buffer, and catalase was eluted with a linear gradient (100 ml total) of 0.01–0.3 M-KPB, pH 7.8. Peak catalase fractions were pooled and concentrated by dialysis against 4 vol. of 100%-saturated $(NH_4)_2SO_4$, pH 7.8, followed by centrifugation at 23500 *g* for 60 min. The pellet was resuspended in approx. 10 ml of 0.2 M-KPB, pH 7.8. The sample was applied directly to a phenyl-Sepharose CL-4B column (1.4 cm × 10 cm) that had been equilibrated previously in 0.2 M-KPB, pH 7.8. After the column had been washed with 100 ml of the same buffer, purified catalase was eluted with a linear gradient (100 ml total) of 0–100% (v/v) ethylene glycol and 0.2–0 M-KPB, pH 7.8. Peak fractions were pooled and vacuum-dialysed to 1.0 ml against 10 mM-Tris/HCl(pH 8.9)/20% (v/v) ethylene glycol.

A 70 $\mu$g portion of purified catalase in 0.2 ml of buffer (see above) was used for initial injections into rabbits as described by Trelease *et al.* [20]. For the third injection, 53 $\mu$g of catalase was used and, 9 days later, 108 $\mu$g of antigen was injected intravenously into a marginal ear vein. Purification of IgGs from anti-catalase or preimmune serum was performed as described in [20].

**Immunochemical analyses**

Immunotitration was performed by incubating 50 $\mu$l of cell-free extract (containing 45 $\mu$kat of catalase prepared as in ref. [19]) with various amounts of anti-catalase, purified IgGs or preimmune serum. Volumes were adjusted to 1 ml with 150 mM-NaCl/50 mM-Tris/HCl(pH 8.9)/3 mM-EDTA/1 mM-PMSF. After incubation at 25 °C for 1 h, followed by 4 °C for 16 h, catalase activity was assayed before and after centrifugation (13000 *g*, 15 min, 4 °C).

Double-diffusion immunoprecipitation was performed as described in [20]. Electrophoretic transfer (Western) blotting was performed by using procedures described previously [19]. Cell-free extracts for SDS/PAGE/Western blotting also were prepared as described in [19].

**RNA isolation**

All glassware was heated at 230 °C for at least 12 h, and aqueous solutions were first treated with 0.1% (v/v) diethyl pyrocarbonate and subsequently autoclaved for 30 min. Total RNA was isolated from cotyledons of 22 h-dark-grown cotton seedlings (radicles 20 mm long) and from embryos collected 28 dpa using the guanidine thiocyanate/CsCl extraction procedure [25]. The homogenizing medium consisted of 4 M-guanidine thiocyanate, 50 mM-Tris/HCl, pH 7.6, 2% (w/v) Sarkosyl, 1% (v/v) 2-mercaptoethanol and included 10 mM-vanadyl ribonucleosides prepared as described in [25]. Poly(A)$^+$ RNA was separated and collected by using oligo(dT)–cellulose chromatography.

**Translation *in vitro***

Just before translation, samples of RNA were heated for 5 min at 65 °C in Microfuge tubes and then rapidly cooled in ice water as described by Rachubinski *et al.* [4]. Cell-free translation was performed for 60 min at 29 °C in a commercial nuclease-treated rabbit reticulocyte lysate system or 90 min in a wheat-germ lysate system with [$^{35}$S]methionine as described by the supplier (Promega–Biotec, Madison, WI, U.S.A.). Optimal trans-

lations were obtained with 2.5 $\mu$g and 5.0 $\mu$g of poly(A)$^+$ RNA in the reticulocyte and wheat-germ systems respectively.

### Immunoprecipitation of catalase *in vitro*

Immunoprecipitation was based on the procedure described by Rachubinski *et al.* [4] with variations given in the legend of Fig. 5 (below). The translation mixture was diluted to 1 ml with Buffer A (4 °C). The diluted translation mixture was chilled in ice/water for 15 min in a Microfuge tube and centrifuged at 13000 *g* for 15 min. The supernatant was adjusted to 1 M-NaCl (50 $\mu$g of solid NaCl) and incubated (1 h, 25 °C) with 20 $\mu$l of Pansorbin [Protein A-positive *Staphylococcus aureus* cells (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.)] prewashed and resuspended in Buffer A lacking the 1 M-NaCl. The mixture was centrifuged for 15 min at 13000 *g*; the sedimented cells were discarded. The supernatant was incubated with 8 $\mu$l of anti-catalase serum or 40 $\mu$g of purified IgGs for 60 min (end-over-end mixing) at 25 °C then overnight at 4 °C. A 20 $\mu$l portion of Pansorbin was added, and the mixture was incubated at room temperature with end-over-end mixing for 1 h. The cells were collected by centrifugation and washed five times in Buffer B, followed by two washings in Buffer B minus detergents. For SDS/PAGE, the immune complexes were dissociated by boiling in 50 $\mu$l of 4% SDS/50 mM-Tris/HCl(pH 6.9)/10%(v/v)glycerol. The cells were removed by centrifugation, the supernatant was adjusted to 10 mM-dithiothreitol, and the proteins were reduced by heating in a boiling-water bath for 15 min. The reduced proteins were then alkylated as described above.

In a modification designed to prevent potential proteolysis [26], catalase synthesized *in vitro* was immunoprecipitated as described above, except that the translation mixture was first adjusted to 2% SDS and heated in a boiling-water bath for 15 min before dilution with Buffer A (see Fig. 5 below, lane P).

In another variation, catalase synthesized *in vitro* was immunoprecipitated directly by incubating the diluted translation mixture with 14 $\mu$l of anti-catalase serum (or 70 $\mu$g of IgGs) for 1 h at 25 °C. Thereafter the mixture was incubated for 1 h at 25 °C and overnight at 4 °C with 2 $\mu$g of purified catalase. The immunoprecipitate was collected by centrifugation (13000 *g*, 15 min, 4 °C); it was then washed, dissociated and alkylated as described above.

### Pulse-labelling *in vivo*

[$^{35}$S]Methionine (20–50 $\mu$Ci per seed) was applied directly to the cotyledons of intact cultured embryos or germinated seedlings, and the organisms were incubated in the dark in a humid environment at 30 °C. Cell-free extracts were prepared as described previously [19], except that the extraction buffer was composed of 100 mM-KPB, pH 7.2, with 0.5% (v/v) Triton X-100, 5 mM-iodoacetamide, 2 mM-EDTA, 1 mM-benzamidine hydrochloride, 1 mM-PMSF. Peroxisomes (glyoxysomes) were isolated as described previously [19]. The postmitochondrial supernatant was clarified at 100000 *g* for 60 min and designated the 'cytosolic' fraction (used for Fig. 9 below). Isolated peroxisomes were lysed by diluting the 2 ml peak-catalase fraction with 3 ml of 0.1 M-Tris/HCl / 150 mM - NaCl / 3 mM - EDTA(pH 7.5) / 2 mM - PMSF. The suspension was clarified at 13000 *g* for 15 min; the supernatant was immunoprecipitated or adjusted to 1% (v/v) Triton X-100 and subjected to rate-zonal centrifugation as described in [20].

Catalase labelled *in vivo* was immunoselected (anti-catalase and Pansorbin) by using procedures similar to those described above for catalase synthesized *in vitro*.

## RESULTS

### Catalase purification

A summary of the procedure for catalase purification from cotyledons of dark-grown cotton seedlings is given in Table 1. After chromatography on hydroxyapatite, the resulting catalase specific activity was 796 $\mu$kat/mg of protein; the $A_{280}/A_{405}$ ratio was 1.0. Because two minor contaminating proteins (subunit $M_r$ just slightly lower than that of catalase) were still detectable on silver-stained gels, the enzyme preparation was subjected to $(NH_4)_2SO_4$ precipitation and hydrophobic chromatography on phenyl–Sepharose CL-4B. Although this last step partially inactivated the enzyme (Table 1), it effectively separated the minor contaminants from catalase (Fig. 1*a*).

### PAGE of purified catalase

When subjected to SDS/PAGE, purified catalase migrated as a single protein band with a subunit $M_r$ of approx. 57000 (Fig. 1*a*). Identical results (one homogeneous band) were obtained whether SDS/PAGE was performed with 7–13%-(w/v)-polyacrylamide gradient or 8% 'non-gradient' gels (results not shown).

**Table 1. Summary of catalase purification from cotyledons (450 pairs) of dark-grown cotton seedlings**

| Step | Procedure | Enzyme activity ($\mu$kat) | Protein (mg) | Specific activity ($\mu$kat/mg) | Yield (%) |
|---|---|---|---|---|---|
| 1 | Acetone extract/homogenate | 9040 | 2030 | 4 | 100 |
| 2 | Ethanol/chloroform fractionation | 9038 | 211 | 43 | 100 |
| 3 | DEAE-Servacel ion exchange | 5900 | 76 | 78 | 65 |
| 4 | Ethanol fractionation | 4730 | 26 | 182 | 52 |
| 5 | 28–55%-Satd. $NH_4SO_4$/dialysis | 2400 | 6 | 400 | 27 |
| 6 | Hydroxyapatite pool | 796 | 1 | 796 | 9 |
| 7 | $(NH_4)_2SO_4$/phenyl–Sepharose | – | 0.4 | –* | – |

* After step 7 catalase was partially inactivated; this was used as antigen.

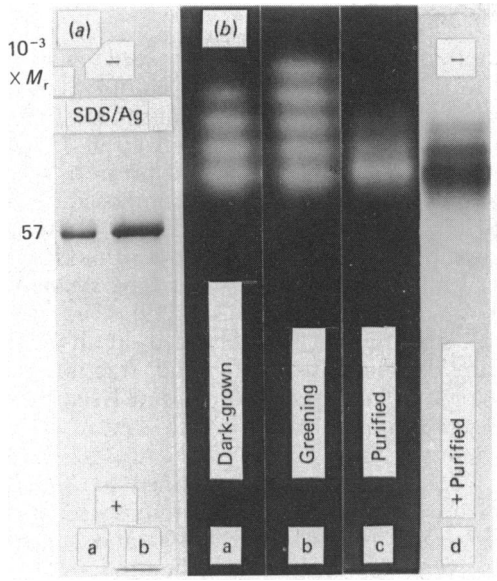


**Fig. 1. Electrophoresis of cottonseed catalase**

(*a*) Silver-stained SDS/10%-(w/v)-polyacrylamide gel (SDS/Ag) (17 cm long) of catalase purified from cotyledons of cotton seedlings; approx. 0.5 $\mu$g (lane a) or 2 $\mu$g (lane b) of protein was applied. (*b*) Non-denaturing polyacrylamide (5%) gels of catalase: lanes a and b, cell-free extracts (13000 ***g*** supernatant from cotyledons powdered in liquid $N_2$, then homogenized in 60 mM-Tris/HCl (pH 6.9)/20% (v/v) glycerol/10 mM-DTT/1 mM-PMSF) of dark-grown (lane a) or greening (lane b) seedlings; lanes c and d, purified catalase. After electrophoresis, lanes a–c were stained for catalase activity; lane d was stained for protein with silver. Approx. 6 nkat of activity was applied to each lane (a–d).

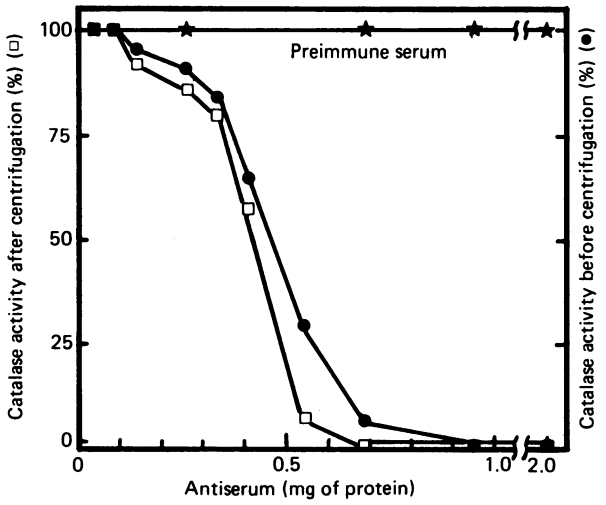


**Fig. 2. Immunotitration of catalase**

Approx. 50 $\mu$l of a cell-free extract (containing 45 $\mu$kat of catalase) from cotyledons of dark-grown cotton seedlings and various amounts of anti-(purified cotton catalase) serum or control (preimmune) serum were diluted to a final volume of 1 ml with 50 mM-Tris/HCl/150 mM-NaCl/3 mM-EDTA (pH 8.0)/1 mM-PMSF. After incubation for 1 h at 25 °C and 16 h at 4 °C, catalase activity was assayed before and after centrifugation to remove antigen–antibody precipitates.

Non-denaturing PAGE of purified catalase (after Step 6 of Table 1) initially gave variable results. Instead of showing four or five resolvable bands, similar to those in Fig. 1*b*, lanes a and b, purified catalase produced a wide, relatively anodic, smear. When the enzyme was purified again with 10 mM-DTT included in all buffers, catalase migrated as three distinct electrophoretic forms (Fig. 1*b*, lanes c and d). Up until Step 4 (Table 1) of the purification procedure, DTT did not adversely affect the enzyme (cf. [11]), but instead, stabilized catalase enzyme activity. After Step 4, however, catalase was only partly (10%) active in the presence of 10 mM-DTT. The inactivation of catalase by DTT was largely reversible, because a significant proportion (> 60%) of the activity was recovered after the DTT was removed by dialysis or non-denaturing PAGE.

### Immunochemical studies

Addition of the appropriate amount of antiserum completely inhibited catalase activity (Fig. 2). Similar results were obtained with IgGs purified from the antiserum (results not shown). Preimmune serum had no effect on catalase activity (Fig. 2).

A single contiguous precipitin line formed after double immunodiffusion between the antiserum and purified catalase, cell-free extracts and isolated peroxisomes (Figs. 3*a* and 3*b*). These results indicate that the antiserum was monospecific to catalase and that catalase does not undergo any significant biochemical alterations during seed maturation, germination or post-germinative growth (Figs. 3*a* and 3*b*). Furthermore, the contiguous precipitin lines formed with purified catalase and a cell-free extract (cf. wells g and e) indicated that catalase did not undergo any major biochemical changes during purification. The antiserum cross-reacted and indicated a partial identity between cotton catalase and catalase from spinach leaves, cucumber, soybean (*Glycine max*) and pumpkin cotyledons (results not shown).

Western blots of SDS/polyacrylamide gels showed only one immunoreactive peptide in cell-free extracts and isolated glyoxysomes from cotyledons of 4-day dark-grown cotton seedlings (Fig. 4, lanes C and E), providing another assessment for monospecificity of the antiserum. Similar results were obtained when the gels/blots were greatly overloaded (results not shown). This peptide possessed the same subunit $M_r$ (57000) as that of purified cotton catalase (lane D). Identical results were obtained with SDS and Triton X-100 extracts (instead of cell-free extracts; see ref. [19], and when SDS/PAGE was performed in 8 or 10% 'non-gradient' gels (results not shown). In contrast, an additional minor immunoreactive polypeptide (59000-$M_r$ subunit) was detected in cell-free extracts of cotyledons from dark-grown (4 days) cucumber and pumpkin seedlings (Fig. 4, lanes A and B). Control blots probed with preimmune serum showed no immunoreactivity (results not shown).

### Translation of catalase *in vitro*

Poly(A)$^+$ mRNA was translated in a commercial reticulocyte lysate or wheat-germ translation system in the presence of [$^{35}$S]methionine (Fig. 5). In all translation experiments a major radioactive protein with a subunit

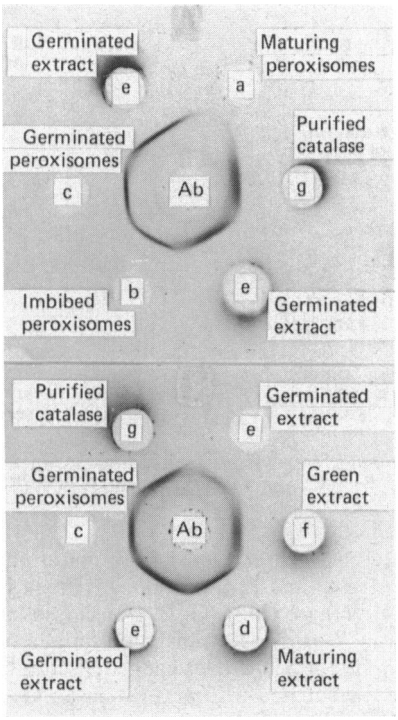


**Fig. 3. Comparison of cotton catalase by double-diffusion immunoprecipitation**

The centre wells contained anti-catalase serum (Ab; 10 $\mu$l of a 1:32 dilution); the outer wells contained isolated peroxisomes from cotyledons of 42-dpa maturing (well a), 6 h-imbibed (well b), and 3-days-dark-grown (well c) seeds; cell-free extracts of cotyledons from 42-dpa maturing (well d), 3-days-dark-grown (well e) and green (well f) seedlings; purified catalase (g). All outer wells (except well a) contained approx. 0.1–0.2 $\mu$kat of catalase. Precipitin lines were stained with Coomassie Blue. Note the monospecificity of the antiserum and complete identity (contiguous lines).

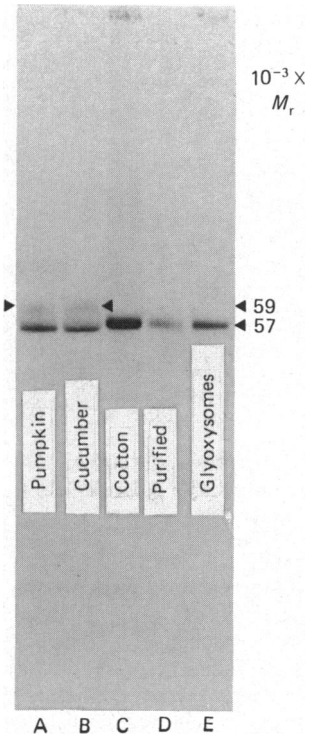


**Fig. 4. Comparison of catalase (subunits) using the SDS/PAGE/Western-blot technique**

SDS/PAGE was performed in 4%-(w/v)-polyacrylamide stacking/7–13% (17 cm long) gradient gels; electroblots were probed with anti-(cotton catalase) serum. Lanes A–C: cell-free extracts from dark-grown (4 days) seedlings of pumpkin (A), cucumber (B), cotton (C); lane D, purified cotton catalase; lane E, isolated glyoxysomes from cotyledons of 3 day-dark-grown cotton seedlings (this lane contained approx. 300 ng of protein).

$M_r$ of 57000 was detected (see, e.g., Fig. 5, lane M). The size of this protein was identical with that of immunoprecipitated peroxisomal catalase labelled *in vivo* (Fig. 5, lane N) and the unlabelled purified enzyme (lane K). In competition experiments, in which an excess of unlabelled purified catalase was added to the translation mixture before immunoprecipitation, the 57000-$M_r$ protein was not detected (Fig. 5, lane L). The electrophoretic mobilities of the translation products from reticulocyte lysates were similar to those from wheat-germ extracts. Similar results were obtained whether RNA was extracted from maturing seeds or from cotyledons of dark-grown germinated seeds, and when samples were subjected to SDS/PAGE in the presence of urea.

The results of translation experiments significantly differed, however, depending on the methodology employed for immunoselection of catalase from the translation products. Only the 57000-$M_r$ protein was detected when translation products were subjected to direct immunoprecipitation, i.e. addition of anti-catalase, followed by unlabelled purified catalase as carrier (Fig. 5, lane M). An additional protein with a subunit $M_r$ of 64000 was detected when translation products were subjected to immunoadsorption, i.e. incubation with anti-catalase overnight at 4 °C, followed by addition of Pansorbin (Fig. 5, lanes B,D,E and G–J). Incubation of translation products with IgGs from preimmune serum and Pansorbin resulted in the absence of both the 57000- and 64000-$M_r$ proteins. The 64000-$M_r$ protein was not detected by immunoadsorption when translation products were incubated with anti-catalase for only 1 h (lane O) or when the translation products were heated in the presence of SDS before the addition of anti-catalase (lane Q), indicating that the 57000-$M_r$ protein was not the result of proteolysis during immunoselection. Both the 57000- and 64000-$M_r$ proteins were detected when translation assays were terminated early (lanes D,G and I), indicating that the 64000-$M_r$ protein was not processed to the 57000-$M_r$ species of catalase in the translation mixture. The ability to detect the 64000-$M_r$ protein only after long incubations with anti-catalase followed by an immunoadsorbent (Pansorbin) indicates that it is a minor translation product, produced in small quantities compared with the 57000-$M_r$ catalase.

### Biosynthesis of catalase *in vivo*

When crude extracts or isolated peroxisomes were immunoselected with anti-catalase, both the 57000- and the 64000-$M_r$ proteins were detected (see, e.g., Fig. 5, lane C). A typical precursor–product relationship between

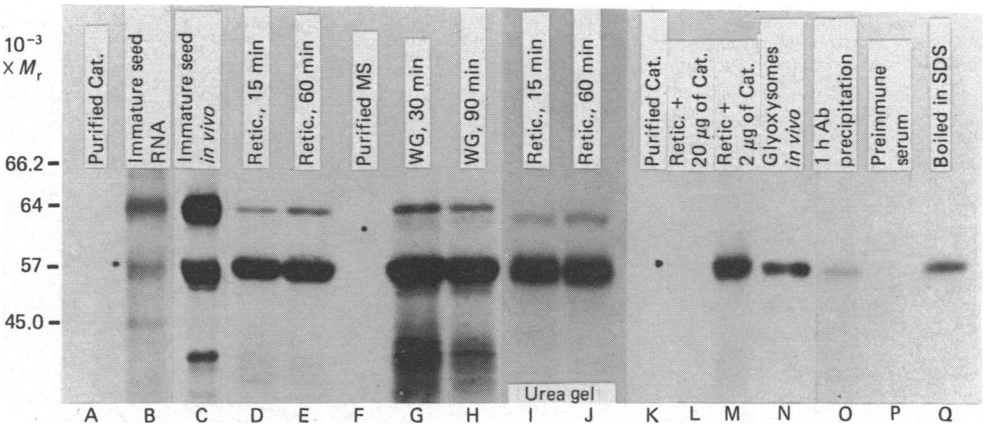


**Fig. 5. SDS/PAGE and fluorography of '*in-vitro*'-synthesized catalase (Cat.) immunoselected from translation products of poly(A)$^+$ RNA added to either a commercial reticulocyte lysate (Retic.; lanes B, D, E, I, J, L and M) or wheat-germ (WG; lanes G, H, and O–Q) system**

A and K, radioactive ink marked position of purified catalase; B, translation with RNA extracted from maturing seeds; C, maturing seeds cultured for 2 days on abscisic acid and labelled *in vivo* for 60 min; D and E, translations terminated after 15 and 60 min respectively; F, radioactive ink marking position of purified malate synthase (MS); G–H, translations terminated after 30 and 90 min respectively; I and J, same as in D and E, but SDS/PAGE in 4 M-urea; L, translation mixture with 20 μg of purified catalase added before anti-catalase; M, translation mixture with 2 μg of purified catalase added after anti-catalase; N, immunoselected glyoxysomal catalase labelled *in vivo*; O, translation mixture incubated with anti-catalase (Ab) for 1 h at room temperature; P, preimmune serum; Q, mixture boiled for 15 min in 2% SDS immediately after translation. All (except O and P) were incubated with purified IgGs (containing anti-catalase) for 1 h at room temperature, then at 4 °C overnight. All (except L–N) were incubated with Pansorbin for 1 h at room temperature.

the 64000- and the 57000-$M_r$ proteins could not be demonstrated by using standard pulse–chase techniques. The 57000-$M_r$ protein was detected in crude extracts of germinated seeds after a 15 min pulse (Fig. 6, lanes A,C and E), whereas both the 57000- and the 64000-$M_r$ proteins were found after a 5 h chase (lanes B and D). Relatively less radioactive catalase was resolved in crude extracts at later stages of seedling growth, owing to the abundance of unlabelled catalase already present (lanes E and F).

When crude extracts were partially purified by rate-zonal centrifugation, newly synthesized monomers (5S) of both the 64000- and the 57000-$M_r$ proteins were detected in maturing and germinated seeds after 30–45 min (Figs. 7*a* and 7*c*). After 60 min labelling, a small amount of the 64000-$M_r$ protein was detected as an aggregated (20S) form, whereas a significant proportion of the 57000-$M_r$ catalase was found as the native (11S) tetramer.

The 64000-$M_r$ protein was never detected on Western blots probed with anti-catalase, even after it was partially purified by rate-zonal centrifugation (Fig. 8). The 64000-$M_r$ protein usually was not visible on the Coomassie Blue-stained SDS-containing gel of immunoprecipitates, but detectable only after fluorography.

Newly synthesized monomers (5S) of both the 57000- and the 64000-$M_r$ proteins were found in the cytosolic fraction after labelling for 45 min (Fig. 9). Only aggregated forms of both the 57000- and the 64000-$M_r$ proteins (11S and 20S respectively) were found in isolated peroxisomes (Fig. 10), even after long (e.g. 22 h) chase periods (results not shown). The aggregated (20S) form of the 64000-$M_r$ protein appears to have some affinity for the peroxisomal membrane, because it was never detected in the cytosolic fraction, but was found in peroxisomal fractions only, even in experiments when up to 80% of the peroxisomes were disrupted (as judged by recovery of catalase activity) (results not shown).

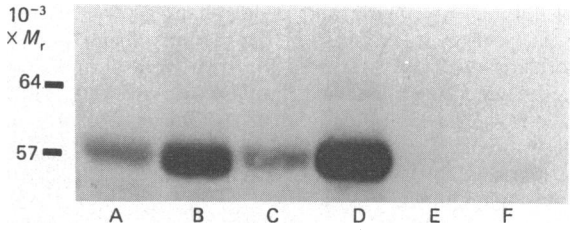


**Fig. 6. SDS/PAGE and fluorography of '*in vivo*'-labelled catalase after a 15 min pulse with [$^{35}$S]methionine (lanes A, C and E) or a 15 min pulse and a 5 h chase with 10 mM unlabelled methionine (lanes B, D and F)**

Label was applied to cotyledons of intact seedlings at 10 (lanes A and B), 18 (lanes C and D) or 34 h (lanes E and F) after imbibition. Tissue was homogenized in 100 mM-potassium phosphate/0.5% Triton X-100/5 mM-iodoacetamide/2 mM-EDTA/1 mM-benzamidine hydrochloride/1 mM-PMSF, pH 7.2, clarified at 13000 *g* for 30 min and subjected to immunoselection with anti-catalase and Pansorbin. The 64000-$M_r$ immunoreactive protein is present as faint bands in lanes B and D.

## DISCUSSION

Catalase was purified from cotyledons of dark-grown germinated cotton seedlings, and monospecific (as judged by Ouchterlony double diffusion and Western blotting) antibodies were raised in rabbits. The specific activity reported here (796 μkat/mg of protein;

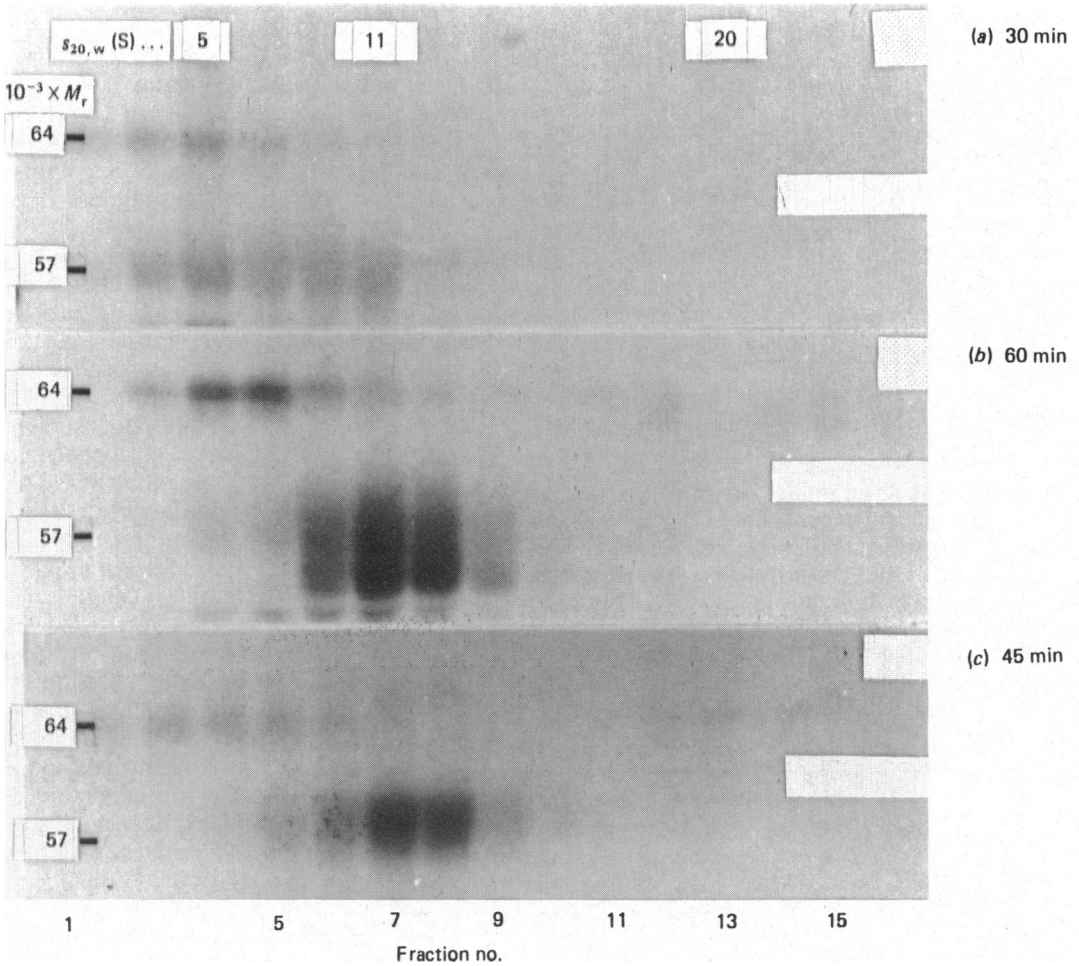


**Fig. 7. Sedimentation analysis of catalase in crude extracts from maturing seeds (panels *a* and *b*) and cotyledons of dark-grown (22 h) seedlings (panel *c*) labelled *in vivo* with [$^{35}$S]methionine for the time indicated**

Maturing seeds (42 dpa) were cultured for 2 days on abscisic acid before radiolabelling. Tissue was homogenized in 100 mM-potassium phosphate/5 mM-iodoacetamide/2 mM-EDTA/1 mM-benzamidine/1 mM-PMSF, pH 7.2, clarified at 13000 *g* for 30 min and subjected to rate-zonal centrifugation in 5–25 %-(w/v)-sucrose (14 ml total). Immunoselected proteins (anti-catalase and Pansorbin) from each gradient fraction (0.6 ml) was subjected to SDS/PAGE and fluorography. The approximate $s_{20,w}$ values given for various fractions correspond to the sedimentation behaviour of haemoglobin (5 S), bovine liver catalase (11 S) and ribulose-bisphosphate carboxylase (20 S) in similar gradients.

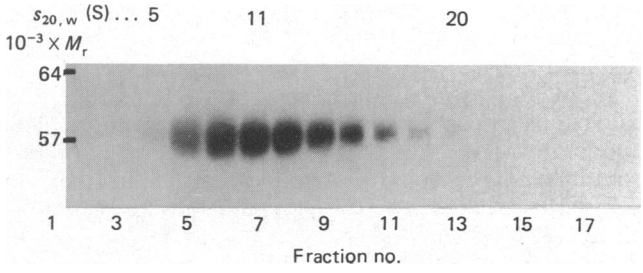


**Fig. 8. Western blot of fractions from a rate-zonal gradient similar to that in Fig. 7(*c*) (germinated seeds)**

A constant amount (50 $\mu$l) of each gradient fraction was subjected to SDS/PAGE; the electroblot was probed with anti-catalase serum. The 64000-$M_r$ anti-catalase-immunoreactive protein was not detected in any region of the gradient.

$5 \times 10^4$ mol/min per mg of protein) is comparable with, or higher than, that reported for catalase purified from other animal, bacterial and plant sources. The $A_{280}/A_{405}$ ratio of 1.0 is comparable with (or lower than) that reported for purified catalase from most other plant sources, but is higher than that (approx. 0.7) reported for animal and bacterial [9] and sunflower [18] catalase.

In a previous paper [19] we reported the existence of multiple forms of catalase in cotton. By using non-denaturing PAGE and isoelectric focusing, five distinct electrophoretic forms were resolved in crude extracts and isolated peroxisomes from germinated seeds. The various forms were shown to be charge isomers, each with an $M_r$ of approx. 230000 [17]. When purified cotton catalase was subjected to non-denaturing PAGE, three distinct electrophoretic forms were resolved (Fig. 1*b*, lanes c and d). These three forms co-migrated with the three most anodic forms in crude homogenates, indicating that two

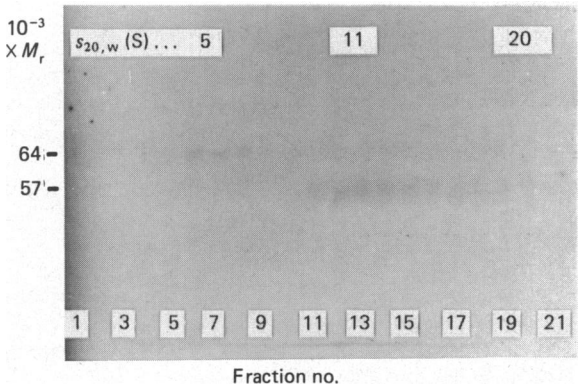


**Fig. 9. Sedimentiation analysis of cytosolic soluble catalase**

Cotyledons of dark-grown seedlings were labelled with [$^{35}$S]methionine for 45 min and chopped to preserve organelles. The soluble fraction (100000 *g* supernatant after differential centrifugation) was subjected to rate-zonal centrifugation in 5–20% (w/v) sucrose (14 ml total). Immunoselected proteins (anti-catalase and Pansorbin) from each fraction were subjected to SDS/PAGE and fluorography. Although difficult to detect after centrifugation of this highly diluted cytosolic fraction, monomeric (5 S) forms of both 64000- and 57000-$M_r$ catalase are apparent.

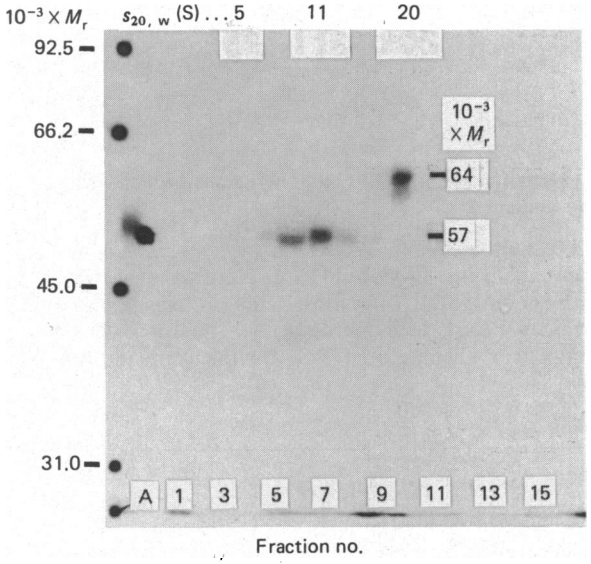


**Fig. 10. Sedimentation analysis of glyoxysomal catalase**

Cotyledons of dark-grown seedlings were labelled with [$^{35}$S]methionine for 24 h. After labelling, lysed glyoxysomes were adjusted to 1% Triton X-100 and subjected to rate-zonal centrifugation in sucrose. Immunoselected proteins were subjected to SDS/PAGE and fluorography. A, lane with radioactive ink marking position of purified catalase.

cathodic forms of catalase were lost (or converted into a more anodic form) during purification. Nevertheless, the antibody to this enzyme preparation detected all forms of catalase in germinated seedlings.

The existence of multiple electrophoretic variants in a preparation of purified catalase has been reported previously for mammalian (e.g. rat [27] and mouse liver [28]) and plant (e.g. spinach [10] and sunflower [18]) sources, and three distinct forms of catalase were individually purified from maize [14]. Other than the catalase isoenzyme system of maize [29], very little information is known regarding the basis of catalase heterogeneity in animals or plants. It appears that heterogeneity of cotton catalase does not represent an artefact of extraction [19], and the existence of multiple forms of catalase in the purified preparation demonstrated here indicates that charge heterogeneity is not due to some catalase-binding protein or some loosely bound small molecule. Purified cotton catalase does not react with Schiff's reagent, specifically bind concanavalin A nor does it become labelled *in vivo* with [$^3$H]glucosamine (results not shown), indicating that cotton catalase is not a glycoprotein.

When analysed by the SDS/PAGE/Western-blot technique (Fig. 4), purified cotton catalase was found to be the same size as catalase in cell-free extracts and isolated glyoxysomes, indicating that the enzyme did not undergo any detectable change in subunit $M_r$ during purification, as was discovered for mammalian catalase [30–32]. Our attempts to detect a larger putative precursor to cotton catalase using this technique have proven unsuccessful. Yamaguchi *et al.* [17] also found only one catalase polypeptide on blots of tissues possessing various types of peroxisomes. In contrast, two different-$M_r$ catalase subunits were reported for maize scutellum [33] and cucumber cotyledons [6,7,34,35], and two immunoreactive catalase polypeptides (subunit $M_r$ 55000 and 59000) were detected on Western blots of three greening cucurbit species [16,17,36]. Confirming this, we observed two immunoreactive catalase polypeptides on Western blots of cucumber and pumpkin seedlings probed with anti-(cotton catalase) serum (Fig. 4, lanes A and B). Therefore a protein analogous to the 59000-$M_r$ polypeptide could not be detected in cotton, even though our SDS-containing gel/Western-blot systems possessed the resolution/capability.

Catalase also appears to be synthesized *in vitro* in a form indistinguishable from the respective native apo subunit in the yeasts *Saccharomyces* [37], *Candida* [38] and *Hansenula polymorpha* [39], in rat liver [3,27,32,40] and in sweet-potato root [41]. The results obtained in the present investigation essentially confirm these latter investigations, suggesting that catalase is initially synthesized in its final form. Synthesis of cotton catalase *in vitro* consistently revealed a predominant protein with a subunit $M_r$ of 57000, the same as that of both the authentic enzyme labelled *in vivo* and the purified enzyme.

A 64000-$M_r$ protein, however, was detected in cotton cotyledons in experiments performed for immunoselection of low-level translation products. Results of our experiments *in vitro* and *in vivo* indicate neither that the 57000-$M_r$ catalase is an artefact resulting from proteolysis of the 64000-$M_r$ protein, nor that the 64000-$M_r$ protein is a precursor possessing a cleavable signal or transit peptide. '*In vivo*' pulse-labelling experiments provided evidence for cytosolic pools of both 64000- and 57000-$M_r$ monomers and incorporation of both proteins into the peroxisome without proteolytic processing. Long-lived species of both proteins were found in the peroxisome as native proteins possessing quarternary structure. Fluorograms of partial proteolysis in SDS-containing gels (Cleveland maps) of the 57000- and

64000-$M_r$ proteins were equivocal for determining whether the 64000-$M_r$ protein is a catalase in cotton. An analogous protein, apparently an aggregated form of catalase also of approx. 20 S, has been reported previously in developing cucumber seeds [34]. The physiological significance, and the unequivocal identity of the 64000-$M_r$ immunoreactive protein as a catalase, remain to be determined.

Overall, the present data are in line with the well-documented evidence for the cytosolic synthesis and post-translational acquisition of rat liver catalase by pre-existing peroxisomes as described in [3,42] rather than by additions initially to the endoplasmic reticulum [42]. The enzyme undergoes no detectable covalent modification during its uptake into peroxisomes and its subsequent maturation to a tetrameric haemoprotein. The mechanism whereby catalase and other proteins [1,2,6,8] are targeted to, and enter, glyoxysomes (peroxisomes) remains to be determined.

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