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# Uricase and Allantoinase in Glyoxysomes[1]

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## ABSTRACT

**In fat-degrading tissues of seedlings of seven different plant species examined, uricase activity (urate:$O_2$ oxidoreductase, EC 1.7.33) was associated with particulate fractions. After equilibrium density centrifugation on sucrose density gradients the enzyme activity was recovered in the glyoxysomal band (density: 1.25 grams per cubic centimeter). Allantoinase is also present in glyoxysomes but, equally, in the proplastid region (density: 1.22 grams per cubic centimeter). Xanthine oxidase, xanthine dehydrogenase, allantoicase, and urease were not detected in glyoxysomes from castor bean endosperm. Uricase in these particles shows its maximal activity at pH 8.9. The apparent $K_m$ is 7.4 $\mu$M. Urate concentrations greater than 120 $\mu$M as well as certain other purine compounds inhibit the enzyme. Cyanide at a concentration of 10 $\mu$M is a potent inhibitor. 2,6-Dichlorophenolindophenol did not substitute for oxygen as electron acceptor.**

The over-all reaction catalyzed by uricase (urate:$O_2$ oxidoreductase, EC 1.7.33) was elucidated by investigations with preparations from hog liver (27), fungi (15, 21), and bacteria (3). As shown in Figure 1, decarboxylation occurs at C-6, and the enzyme functions as an aerobic dehydrogenase. Highly purified preparations have been obtained from hog liver (28).

Early cell fractionation studies with rat liver showed that urate oxidase sedimented with the crude mitochondrial fraction (38). Subsequently, by differential and equilibrium density centrifugation, the enzyme was shown to be associated with other oxidases that produce $H_2O_2$ (L-$\alpha$-hydroxy acid oxidase, D-amino acid oxidase) and catalase in the class of microbody known as peroxisomes (4, 11). Urate oxidase is in fact used as a marker enzyme for these organelles and has been shown to be present in peroxisomes from rat liver (11) and from liver and kidney of birds and amphibians (41) as well as in peroxisomes from certain protozoa (30–32).

The first report of urate oxidase activity in higher plants dates back to 1920 when is was shown that soybean meal was able to degrade uric acid (33). Fosse and his collaborators (13, 14) demonstrated urate oxidase activity in numerous seedlings, and Franke *et al.* (15) and Green and Mitchell (21) studied the enzyme from fungal cells. In addition to its role in the catabolism of purines, as in the extensive digestive process in storage tissue of germinating seedlings, urate oxidase seems to be important in nitrogen mobilization in those vascular plants where allantoin and allantoic acid are major transport forms (36, 37). Schlee and Reinbothe (39) invoked urate oxidase to account for the demonstration that in tissues from such plants uric acid is an intermediate in the synthesis of allantoin from glycine.

However, despite the known and widespread occurrence of urate oxidase in plant tissues, little appears to have been established concerning its properties and intracellular distribution (44). In the present paper we describe properties of the enzyme from the endosperm of germinating castor bean seedlings and its subcellular localization in glyoxysomes from this and other fat-degrading tissues.

## MATERIALS AND METHODS

**Plant Material.** Leaves from tobacco (*Nicotiana tabacum*) and corn (*Zea mays*) were cut from young plants grown in the greenhouse. Sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), and corn seeds were sterilized with 0.5% sodium hypochlorite (Clorox) solution for 20 min, soaked in distilled water for approximately 5 hr, and germinated on moist paper towels at 30 C in the dark for 5 to 6 days.

Seeds of the one-leaved pinyon (*Pinus monophylla*) were collected in the Panamint Mountains (SW Death Valley) and of the Joshua tree at the Walker Pass (California). After sterilization with 10% Clorox solution for 20 min, the seeds were soaked for 8 to 10 hr in distilled water and germinated in moist vermiculite at 30 C and 90% relative humidity in the dark for 5 days (pinyon) or 9 days (Joshua tree). Seeds of castor bean (*Ricinus communis*) were germinated as described previously (20).

**Preparation of Cellular Organelles.** One general grinding procedure was employed with slight modifications for the different plant materials. Usually 2 to 3 ml of grinding medium (described by Gerhardt and Beevers [19, 20], but with the EDTA concentration reduced to 0.001 M), was used for each gram of tissue. All preparations were carried out at 0 to 5 C.

The plant part or tissue of interest was either minced with a razor blade in grinding medium (tobacco and corn leaves, roots, safflower and sunflower cotyledons, yucca perisperm) or chopped in a household onion chopper (corn scutella, pine megagametophytes, castor bean endosperm). The slurry was then ground in a mortar, and the homogenate was passed through four layers of cheesecloth. After centrifuging at 270*g* for 10 min the supernatant solution (supernatant I) was decanted and centrifuged for 30 min at 11,000*g*. This produced supernatant II and a pellet, the crude particulate fraction, which was gently resuspended in grinding medium. Two milliliters of this suspension, corresponding to 5 to 10 g fresh weight of tissue, were layered on stepped or continuous sucrose gradients (20) and centrifuged for 4 to 5 hr at 64,300*g* in a Spinco rotor SW25.2. Discontinuous gradients were fractionated by puncturing the bottom of the tube and collecting separately the visible glyoxysomal, proplastid, and mitochondrial bands. An ISCO density gradient fractionator model D was used to collect successive 1-ml samples from continuous gradients.

**Biochemical Assays.** Spectrophotometric assays were con-

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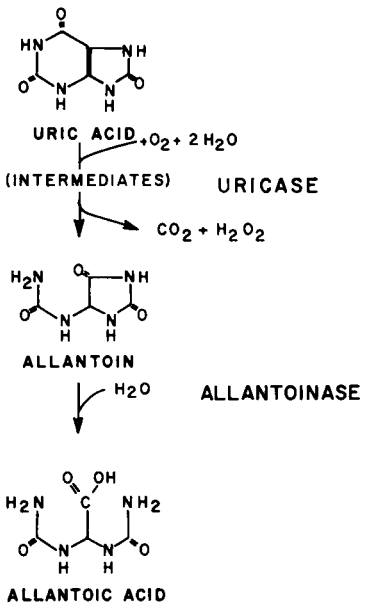


FIG. 1. Enzymatic degradation of uric acid to allantoic acid.

ducted in a Zeiss PMQ II or Beckman DU spectrophotometer attached to a Gilford 2000 recorder.

Uricase activity was assayed by determining the decrease in absorbance at 292.5 nm due to enzymatic oxidation of uric acid. The method used was a modification of the assay described by Müller and Møller (31). The assay mixture contained in a total volume of 1.0 ml of 0.1 M Tricine buffer, pH 8.9; 120 $\mu$M urate (prepared fresh every day in buffer); and 100 to 300 $\mu$g of protein. After preincubation of the protein in buffer for 3 to 5 min the reaction was started by addition of the urate and followed for at least 8 min. Triton X-100 was virtually without effect on the enzyme activity. The extinction coefficient for uric acid was assumed to be $1.22 \times 10^4$ $cm^{-1}$ $M^{-1}$ (31).

For the determination of catalase activity the spectrophotometric assay of Lück (26) was used. Isocitrate lyase was assayed by the method of Dixon and Kornberg (12). Fumarase activity was assayed by a modification of the method of Racker (35). Allantoinase and allantoicase activity were assayed by a differential glyoxylate analysis essentially as described by Trijbels and Vogel (45).

Urease was assayed by the determination of $^{14}CO_2$ released from urea-$^{14}C$ by the enzyme. In a 25-ml Erlenmeyer flask the incubation mixture contained 0.1 M phosphate buffer, pH 7.0, and 0.05% (w/v) bovine serum albumin. A small vial with 0.5 ml of hydroxide of hyamine 10-X (Packard) was placed in the flask, which was then sealed with a serum cap and evacuated. Urea-$^{14}C$ (250 $\mu$moles, containing 40,000 cpm) and subsequently the enzyme solution were injected to give a total volume of 1 ml, and the reaction was allowed to proceed for 25 min. Then 0.5 ml of 1 N HCl was injected to stop the reaction and free the $^{14}CO_2$. After 20 more min of incubation the hyamine solution was carefully transferred into a scintillation vial, and the $^{14}C$ content was measured in a Beckman scintillation counter. Protein was determined by the method of Lowry *et al.* (25); the appropriate corrections for the interference of high sucrose concentrations were made according to Gerhardt and Beevers (18).

## RESULTS

Crude extracts (supernatant I) of endosperm tissue from 5-day-old castor bean seedlings degrade urate at a rate of some 120 nmoles per min per ml. As shown in Table I, 90% of the enzyme activity sediments during centrifugation at 11,000*g*. Uricase from tissues from other fatty seedlings was similarly associated with the crude particulate fraction, although in preparations from corn scutella and safflower cotyledons two-thirds of the activity appeared in the supernatant solution.

When the crude particulate fractions from peanut cotyledons and castor bean endosperm were resolved by equilibrium density centrifugation the enzyme distribution shown in Figure 2 was obtained. The main peak of uricase activity coincides with that of the glyoxysome markers catalase and isocitrate ly-

Table I. *Distribution of Uricase Activity in Tissue Extracts*

| Tissue | Uricase Activity | |
|---|---|---|
| | Crude particulate fraction | Supernatant II |
| | *nmoles urate oxidized per min per 10 g tissue* | |
| Castor bean (endosperm) | 173 (90%) | 19 (10%) |
| Peanut (cotyledons) | 89 (57%) | 67 (43%) |
| Corn (scutella) | 28 (37%) | 48 (63%) |
| Safflower (cotyledons) | 40 (31%) | 89 (69%) |

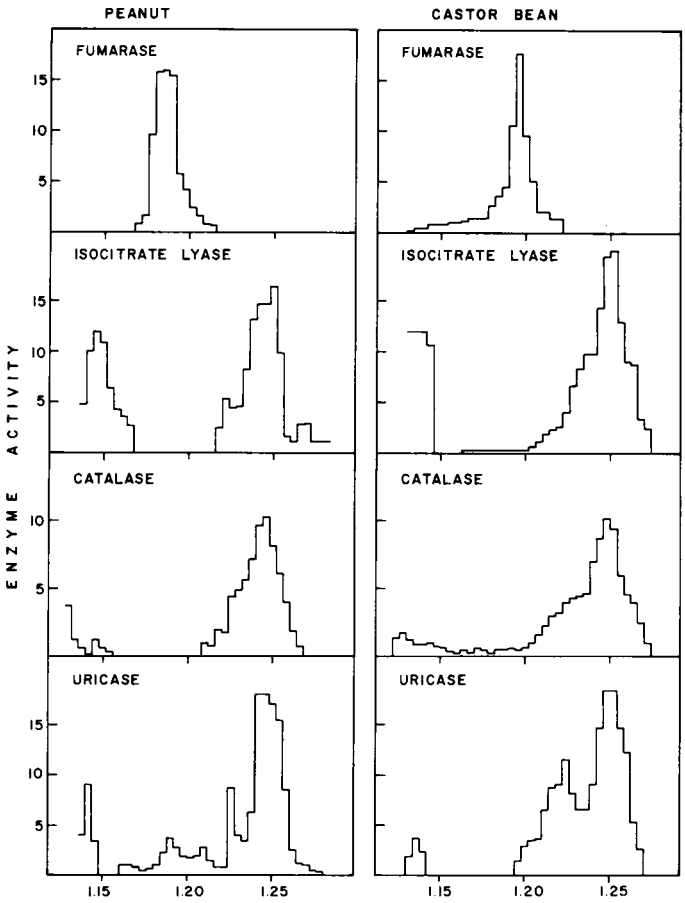


FIG. 2. Isopycnic density gradient centrifugation of crude particulate fractions from peanut cotyledons and castor bean endosperm. Enzyme activities are given in the following units: fumarase: $\mu$moles fumarate formed per min per ml (× 0.07); isocitrate lyase: $\mu$moles glyoxylate formed per min per ml (× 0.05); catalase: $\mu$moles $H_2O_2$ destroyed per min per ml (× 0.001); uricase: nmoles urate oxidized per min per ml.

Table II. *Uricase Activity in Particulate Preparations from Different Plant Sources*

| Plant | Tissue | Fraction[1] | Uricase Activity[1] |
|---|---|---|---|
| | | | *nmoles urate oxidized per min per 10 g tissue* |
| Castor bean | Endosperm | G | 79.0 |
| Peanut | Cotyledons | G | 58.9 |
| Joshua tree | Perisperm | G | 34.4 |
| Corn | Scutella | G | 24.4 |
| Sunflower | Cotyledons | G | 22.3 |
| Safflower | Cotyledons | G | 9.9 |
| One-leaved pinyon | Megagametophyte | CP | 61.8 |
| Castor bean | Roots | CP | 9.3 |
| Corn | Roots | CP | 6.3 |
| Tobacco | Leaves | CP | 0 |
| Corn | Leaves | CP | 0 |

[1] G: Glyoxysomal fraction sedimenting at $\rho$ = 1.25 g/cm$^3$ on a sucrose density gradient; CP: crude particulate fraction (see "Materials and Methods").

Table III. *Enzymes of Purine Catabolism Found in Glyoxysomes of Germinating Castor Bean Seeds*

| Enzyme | Specific Activity |
|---|---|
| | *nmoles substrate utilized per min per mg protein* |
| Xanthine oxidase | ...[1] |
| Xanthine dehydrogenase | ...[1] |
| Uricase | 19.5 (±5) |
| Allantoinase | 2.0–10.5 |
| Allantoicase | ...[1] |
| Urease | ...[1] |

[1] Not detectable.

Table IV. *Distribution of Allantoinase in Endosperm of Germinating Castor Beans*

| Fraction | Relative Specific Activity |
|---|---|
| Crude particulate fraction | 100 |
| Supernatant II | 5.1 |
| Sucrose density gradient | |
| Mitochondria | 0 |
| Proplastids | 212 |
| Glyoxysomes | 246 |

ase at a density of 1.25 g/cm$^3$ and is clearly separated from the sharp peak of fumarase activity at density 1.19 g/cm$^3$ (*i.e.*, the mitochondria). From castor bean preparations, a minor peak of uricase activity was always found in the region of the gradient (density: 1.22 g/cm$^3$) from which proplastids are recovered (7). Similar results were obtained with preparations from peanut cotyledons except that small amounts of the enzyme were recovered throughout the upper parts of the gradient. Several plant tissues from which glyoxysomes have previously been extracted were examined, and uricase was shown to be present in the separated glyoxysomes (Table II). The fatty tissues of yucca and safflower seedlings also yielded clear glyoxysome bands, and here also urate oxidase was found in association with catalase and isocitrate lyase. In preparations from sunflower cotyledons and corn scutella only 30% of the particulate urate oxidase was recovered with the glyoxysomes; activity was present in the mitochondrial and proplastid regions of the gradient. It is very likely that with improved grinding conditions designed especially for these individual tissues the percentage recovery of urate oxidase in the glyoxysomes might be improved; urate oxidase in other regions of the gradient was not due to contamination with intact glyoxysomes since it was not accompanied by isocitrate lyase. No urate oxidase was detected in crude particulate fractions prepared from leaves known to contain peroxisomes, in confirmation of Tolbert's observation (43). Low but significant activity was present in particulate preparations from roots.

Uricase is easily solubilized from castor bean glyoxysomes. The organelles are recovered from the gradient in 54% sucrose. On diluting to 45% sucrose and centrifuging, two-thirds of the uricase activity appeared in the supernatant solution. In this respect the uricase behaves differently from that in liver peroxisomes where it remains particulate even after extensive washing or treatment with detergents (22, 46).

**Other Enzymes of Purine Catabolism.** Tests for other enzymes of the purine breakdown sequence in the glyoxysomes from castor bean endosperm yielded the information given in Table III. Despite several attempts to demonstrate the degradation of xanthine by glyoxysomal preparations, no such enzyme activity could be detected. There was no change in absorbance at 292.5 nm when up to 300 $\mu$g of glyoxysomal protein were incubated in different buffers in the range pH 7 to 9. The addition of FAD or FMN had no effect, and no reduction of NAD occurred. Furthermore, these organelles cannot bring about the cleavage of allantoic acid at measurable rates and no urease activity could be detected even when the test was made by a sensitive assay with urea-$^{14}$C. Allantoinase, the enzyme which hydrolyzes allantoin, the final product of urate oxidation, is present in particulate preparations, as shown earlier by Ory *et al.* (34). The enzyme appears to be rather unstable since only 15 to 20% of the allantoinase activity applied to a sucrose gradient was recovered after 5 hr of centrifugation. Nevertheless, it is clear that part of this activity is associated with the glyoxysomes (Ref. 2 and Table IV). However, roughly half of the enzyme activity recovered from the gradient was present in the proplastid region. Thus, while the glyoxysome in castor bean endosperm appears to be the major or sole particulate location of uricase the next enzyme in the sequence, allantoinase, does not seem to be confined to this organelle.

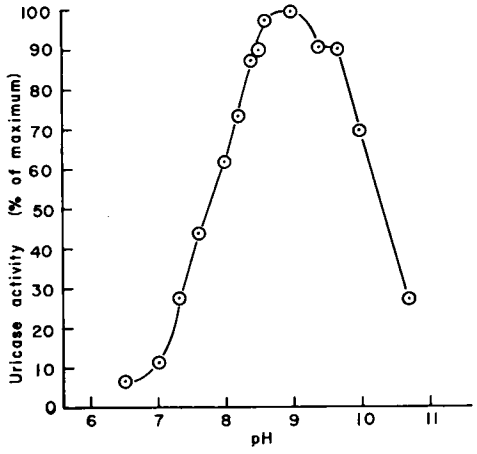


FIG. 3. Uricase activity in glyoxysomes from castor bean endosperm as a function of pH. The buffer used was tris-glycine, adjusted with NaOH or HCl.

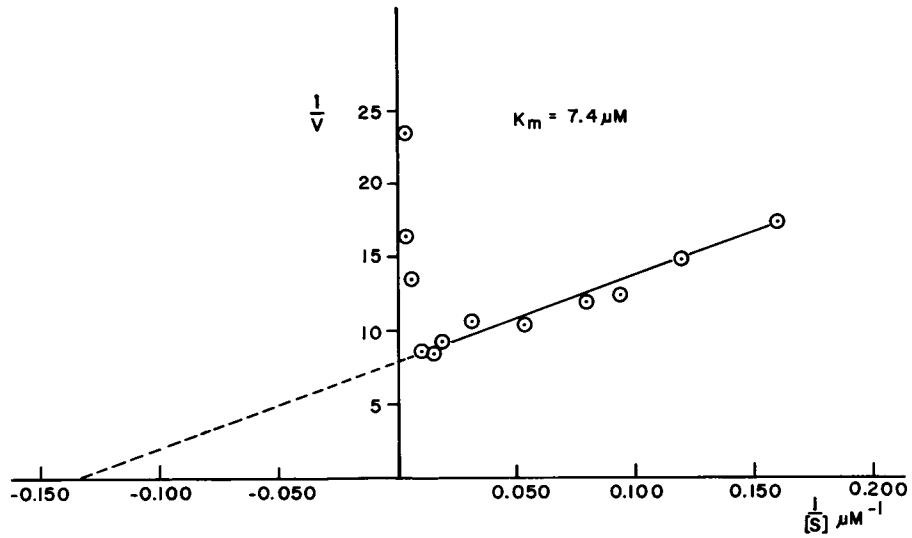


FIG. 4. Effect of substrate concentration on uricase activity in glyoxysomes from castor bean endosperm (Lineweaver-Burk plot).

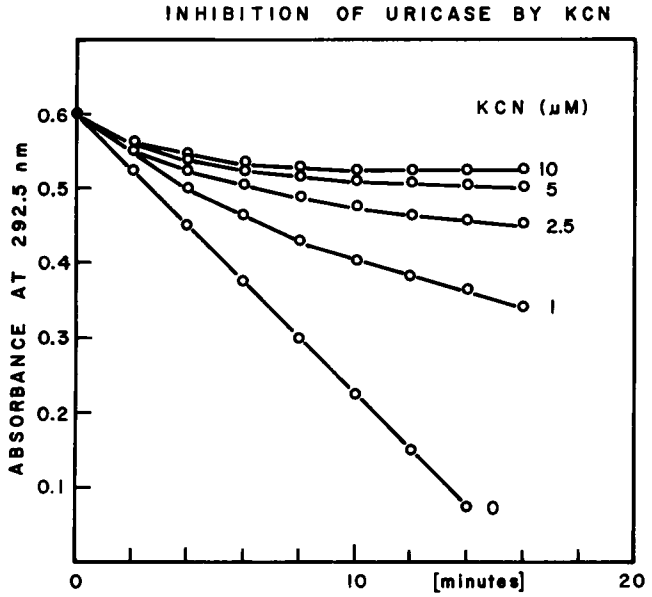


FIG. 5. Inhibition of uricase by cyanide. KCN to give the concentrations shown was added to the standard incubation mixtures at time zero.

**Properties of Glyoxysomal Uricase.** In the isolated glyoxysomes the specific activity of uricase is some 10 times that found in the supernatant I; the organelle preparation was used directly to investigate properties of the enzyme. The oxidase shows activity over a rather broad range of pH (Fig. 3) with an optimum at pH 8.9, close to that reported previously for a crude soybean enzyme (8). The apparent K*m* (7.4 μM) shows that the castor bean enzyme has a high affinity for its substrate (Fig. 4). As with enzymes from other sources (31, 32) the oxidase is progressively inhibited by its substrate as the urate concentration is increased above 120 μM.

The castor bean uricase is extremely sensitive to cyanide (Fig. 5). At a concentration of 10 μM the enzyme was totally inhibited after a lag of 6 to 8 min; at lower concentrations of cyanide this lag period was more prolonged and the degree of inhibition proportionately less.

Figure 6 shows that removing $O_2$ (by bubbling pure $N_2$ through the reaction mixture) prevented the enzymatic breakdown of urate. Oxidation began when air was passed through the cuvette, and a higher rate was brought about when $O_2$ was bubbled briefly through the reaction mixture (24). This sensitivity to $O_2$ concentration is that expected from a flavin nucleotide-linked oxidase. However, the addition of FAD or FMN to incubation mixtures did not increase the reaction rate, and there was no inhibitory effect of blue light in the presence of flavin nucleotides such as that reported with purified hog liver enzyme (40). 2,6-Dichlorophenolindophenol did not substitute for $O_2$ as electron acceptor.

Uricase from mammalian tissues is known to be competitively inhibited by some purine derivatives (27), and the castor bean enzyme reacts similarly. As shown in Table V, the addition of xanthine strongly affects oxidation, hypoxanthine is much less inhibitory, and adenine is without effect.

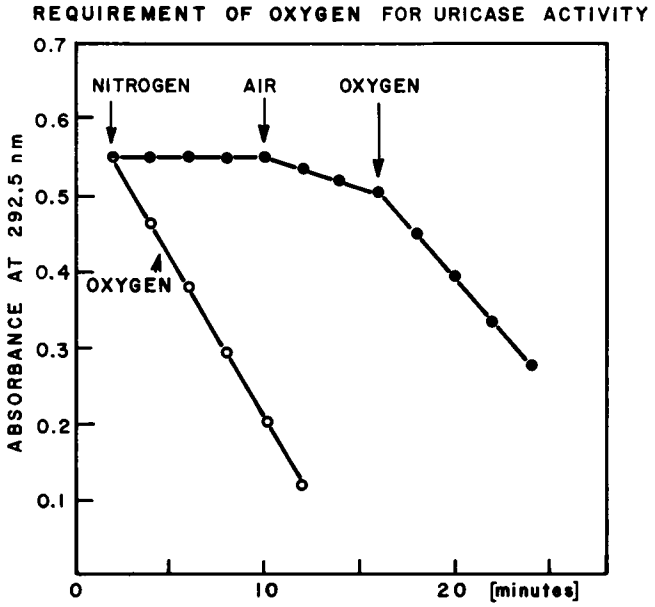


FIG. 6. Requirement of oxygen for uricase activity. ○: control, pure oxygen bubbled through the incubation mixture for 10 sec initially; ●: at the times indicated by arrows, nitrogen, air, and oxygen, respectively, were passed through the reaction mixture for 10 sec.

Table V. *Inhibition of Uricase by Purine Compounds*

| Compound | Concentration | Inhibition |
|---|---|---|
| | μM | % |
| Xanthine | 33.5 | 14 |
| | 67.0 | 40 |
| | 167.0 | 60 |
| Hypoxanthine | 67.0 | 17 |
| Adenine | 67.0 | 5 |

## DISCUSSION

It has been shown previously that glyoxysomes, a type of microbody present in all fatty seedling tissues so far examined, play an important role as the site of the reactions of the glyoxylate cycle (5–7). Incidental to this particular function is the fact that these organelles contain glycolate oxidase and also catalase (7), which probably functions in the β-oxidation sequence in the glyoxysomes (9, 23). These two enzymes have been found in microbodies isolated from many kinds of cells and are considered by de Duve to be distinctive enzymes of the class of microbodies named peroxisomes (11). Uricase is another enzyme which has been found in most of the peroxisome preparations so far described (4, 22, 30–32, 41). It is therefore of interest that, as shown in these investigations, uricase is also present in glyoxysomes. The distribution profile on the sucrose density equilibrium gradient follows faithfully that of isocitrate lyase and catalase. Furthermore, in shallow gradients, the sedimentation velocity of uricase is not significantly different from that of other glyoxysomal enzymes, and preliminary electrophoretic separations have not revealed differences in behavior. Thus, although it is still conceivable that the sharp protein peak that we isolate as glyoxysomes and which shows only one obvious type of organelle when examined in the electron microscope (7) is nevertheless comprised of more than one type of microbody, the evidence in hand at present does not allow this conclusion. We assume, therefore, that each of the enzyme activities specifically associated with the protein band of density 1.25 g/cm$^3$ occur in each of the organelles.

The finding of uricase in glyoxysomes may be thought to support de Duve's suggestion that this organelle represents an ancestral type of microbody which in the course of evolution has lost many of its enzymatic functions (10). However, it is clear that, although the enzyme is present in all of the glyoxysomal preparations examined, it is not an infallible marker for plant microbodies since it is not present in preparations from leaves (Table II and Ref. 43). Uricase is also absent from peroxisomes from certain animal tissues (42).

The facts that uricase activity in the endosperm of 5-day-old castor bean seedlings is quantitatively recovered in the crude particulate fraction and that virtually all of this is recovered, in turn, in the glyoxysomes must mean that these organelles play a role in any purine breakdown occurring in the tissue by the pathway in Figure 1. It seems equally clear that, at most, only one additional step in the sequence, that catalyzed by allantoinase, occurs in these organelles. A similar finding has been made for amphibian hepatic peroxisomes (41, 48).

There is evidence (22, 46) that uricase is present in the crystalline core seen in peroxisomes in sections of liver. Similar cores occur in glyoxysomes in fatty seedlings, but the available evidence supports the suggestion that catalase is the crystalline component (17, 47). This contention is supported by analogy with the situation in microbodies in leaves which apparently do not have urate oxidase but show remarkably extensive crystalline inclusions (16, 29). Furthermore, the ease with which uricase is solubilized from glyoxysomes upon their deliberate breakage renders an association with any crystalline core rather unlikely.

The properties of the uricase from castor bean endosperm are in general similar to those of enzymes from mammals and protozoans studied in detail by previous investigators (27, 28, 31, 32). In distinction from the fungal enzyme (15), the castor bean oxidase is extremely sensitive to cyanide and is unable to use 2,6-dichlorophenolindophenol as electron acceptor.

**Note Added in Proof.** During a re-examination of enzyme distribution in leaf cells, uricase activity has been demonstrated in isolated peroxisomes (T. Huang, R. R. Theimer, unpublished data). Resolution of crude particulate fractions from spinach leaves on continuous sucrose density gradients provided a clear protein band, free of chlorophyll, at a density of 1.235 to 1.250 g/cm$^3$. This band contained the marker enzymes of leaf peroxisomes (catalase and glycolate oxidase) and 75% of the uricase activity from the gradient. The specific activity was 14.9 nmoles urate oxidized per min per mg protein. Wheat leaf peroxisomes (obtained from J. Feierabend) also have uricase activity.

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