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LOCALIZATION OF ENZYMES OF PURINE DEGRADATION IN PLANTS AND ANIMALS

By

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ABSTRACT

LOCALIZATION OF ENZYMES OF PURINE DEGRADATION IN PLANTS AND ANIMALS

by

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Compartmentation plays an essential role in purine degradation in both animals and leguminous plants such as soybean (Glycine max L. Merr.). Nitrogen fixed by symbiotic bacteria within the root nodules of these plants is transported to the green shoot of the plant in the form of allantoin and allantoic acid. These compounds are intermediates in purine degradation.

Two enzymes required for purine degradation, uricase and catalase, were localized within peroxisomes isolated from soybean nodules by isopycnic sucrose density gradient centrifugation. Xanthine dehydrogenase was found in the soluble fraction and allantoinase activity was associated with the endoplasmic reticulum. The purine synthesis pathway has been localized in the plastid fraction from soybean nodules.

The metabolic conversion of symbiotically fixed nitrogen into allantoin and allantoic acid is not only compartmented within several intracellular organelles, but is also divided between two different cell types. Uninfected cells from nodules were separated from cells infected with bacteria on a sucrose step-gradient. The peroxisomal enzymes, uricase and catalase, were associated only with the uninfected cell fraction from soybean nodules. Allantoinase also had a greater specific

activity in the uninfected cell fraction, as did several enzymes whose products are required for purine synthesis, including phosphoglycerate dehydrogenase, aspartate aminotransferase, 6-phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase. Although nitrogen fixation occurs only in the infected cells of the soybean nodule, these data indicate that at least the final reactions occur in the peroxisomes and endoplasmic reticulum of the uninfected cells.

Further degradation of allantoin and allantoic acid must take place in soybean leaves where nitrogen is needed for growth of the shoot. Allantoinase from soybean leaves and germinating seedlings was localized in the endoplasmic reticulum. No allantoicase activity was found in soybean leaves. Allantoin was degraded by seedlings in vivo.

Purine degradation was also investigated in fish liver. Allantoinase activity was present only in the soluble fraction, while uricase and allantoicase were localized in the peroxisomal fraction.

TO MY PARENTS

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INTRODUCTION

Peroxisomes and glyoxysomes, subcellular organelles enclosed by a single lipid bilayer membrane, usually contain flavin oxidases, catalase, and other enzymes participating in catabolic metabolism (1). The term microbody has been previously used to describe all uncharacterized small organelles which contain catalase activity and are found in most eucaryotic cells. The term peroxisome is now recognized to include organelles which are characterized by the presence of catalase and/or flavin oxidase activity, and the term glyoxysome may be used if any part of the glyoxylate cycle is present (2). There are numerous examples of induction of peroxisomal biogenesis, including yeast growth on methanol (3) or alkanes (4), and rats treated with the drug clofibrate (5).

There are currently three recognized types of plant peroxisomes: leaf peroxisomes, glyoxysomes, and unspecialized peroxisomes (6). Leaf peroxisomes contain the glycolate pathway in which glycolate, a component of photorespiration, is converted to glycine (1). The glycerate pathway, also found in leaf peroxisomes, allows the reversible interconversion of serine and glycerate (1). Plants with higher levels of photorespiration (C_3 plants) have greater numbers of leaf peroxisomes than do the C_4 plants, in which the level of photorespiration is much lower, and peroxisomes are observed primarily in the bundle sheath cells (7).

Glyoxysomes, found in the fat-storing cells of germinating fatty seeds, are responsible for the conversion of stored triglyceride to C_4 acids, via the glyoxylate pathway (8). The reactions of the glyoxylate pathway found in glyoxysomes are not duplicated in the mitochondria, but

represent a separate source of C_4 acids for energy and gluconeogenesis. Glyoxysomes are also the exclusive site of beta-oxidation of fatty acids in seedling tissues (8). The question remains whether glyoxysomes become leaf peroxisomes as the tissue greens, or if leaf peroxisomes represent a new population of organelles.

Nonspecialized peroxisomes are found in many non-green plant tissues, including roots (9) and potato tubers (10). The nonspecialized peroxisomes contain high levels of catalase and low levels of other peroxisomal enzymes such as glycolate oxidase and uricase (9, 10). The number of nonspecialized peroxisomes per cell is generally low, and no major metabolic role has been characterized, other than the function of catalase in detoxification of hydrogen peroxide. It is possible that specialized peroxisomes arise from nonspecialized precursors during cellular differentiation.

Liver peroxisomes contain many of the same enzymes and metabolic pathways present in plant peroxisomes (1). Beta oxidation occurs in both peroxisomes and mitochondria of liver cells, but the two pathways differ in many important aspects (1).

The various enzymes and pathways found in peroxisomes from different tissues or developmental stages of the same organism indicate that peroxisomes may be specialized for carrying out unique vital functions in cellular differentiation or in response to environmental stimuli. As outlined above, peroxisomes play an important role in such processes as photorespiration, conversion of stored fats to sucrose (glyoxylate cycle), beta oxidation, methanol oxidation, and detoxification of hydrogen peroxide. The enzymatic content or number of peroxisomes per cell may vary widely between tissues, for example, liver versus kidney in

animals, or mesophyll versus bundle sheath cells in C_4 plants (1). The topic of this thesis is the role peroxisomes play in the degradation of purines in both animals and plants, and the importance of this function to nitrogen metabolism in many economically important nitrogen-fixing legumes.

Purine Catabolism

The nitrogenous excretory products resulting from purine degradation differ between animal phyla due to the loss through evolution of genes for enzymes catalyzing the latter steps of the pathway (11). The respective intermediates in purine degradation are hypoxanthine, xanthine, uric acid, allantoin, allantoic acid, and finally, glyoxylate and urea (Figure 1). The final enzyme of purine degradation present in primates, birds, and some reptiles is xanthine oxidase, which oxidizes both hypoxanthine and xanthine to uric acid, the excretory product. Most mammals, reptiles, and mollusks excrete the next intermediate, allantoin, the product of the uricase reaction. Allantoinase catalyzes the hydrolysis of allantoin to allantoic acid, which is excreted by some fishes. Allantoic acid is hydrolyzed to urea and glyoxylate by allantoicase, the final enzyme present in most fish and amphibians. The aquatic invertebrates retain urease activity, and therefore excrete ammonia.

Xanthine oxidase was found only in the soluble fraction in rat (12) and fish liver (13), but avian xanthine dehydrogenase was reported to be peroxisomal (14). Xanthine dehydrogenase is reportedly the native form of xanthine oxidase (15). Uricase has been found in peroxisomes from mammals (16), fish (13), amphibians (14), and plants (17). Catalase,

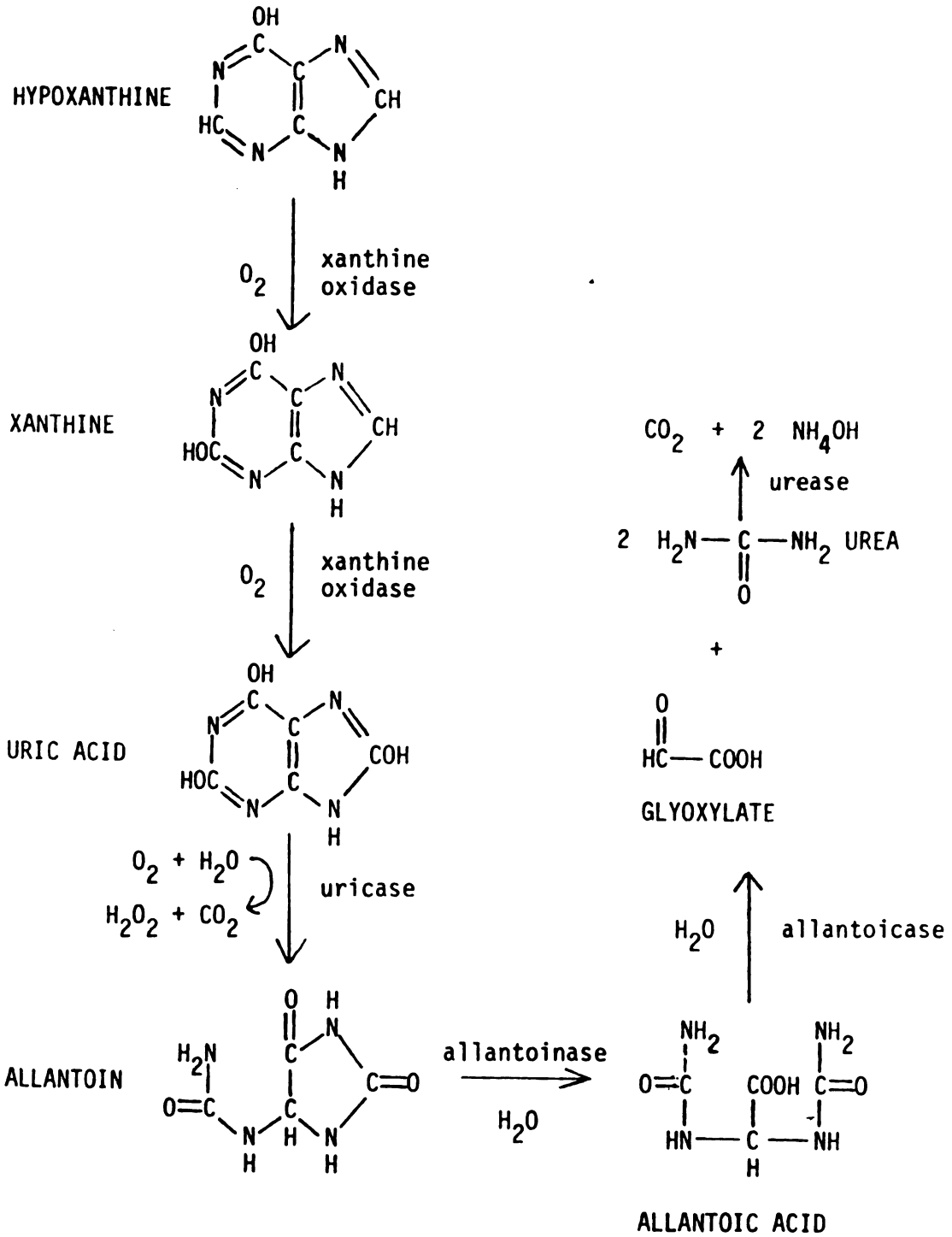

Figure 1. Purine Degradation

which degrades the H_2O_2 produced in the uricase reaction, is also found in peroxisomes (1). Allantoinase has been reported in amphibian and fish liver peroxisomes (14, 13), and at low levels in glyoxysomes from castor bean endosperm (17). However, an equal amount of activity was associated with the proplastid fraction from castor bean endosperm, and eighty percent of the original activity was lost on the gradient (17). In another report on castor bean endosperm allantoinase, most of the activity was found in fractions of density 1.21 g/cc or greater, but the activity was very low, and the location of other organelle fractions was not adequately characterized (18). Allantoicase has been localized in fish liver peroxisomes (13).

Thus some of the enzymes in the pathway of purine degradation have been reported in peroxisomes from a few tissues. This thesis contains a more extensive investigation of purine degradation and the subcellular distribution of the associated enzymes, especially in the plant tissues.

Ureide Metabolism

Two intermediates in purine degradation, allantoin and allantoic acid, are found at high levels in certain nitrogen-fixing legumes (19, 20), and are collectively termed the ureides. Although the presence of relatively high levels of ureides in some plants was reported in 1930 (21) and several workers had suggested purine catabolism resulted in allantoin accumulation as reviewed by Mothes (22), the significance of these compounds in relation to nitrogen fixation in the nodules of certain legumes has been recognized only in the last five years. Matsumoto *et al.* (23) have reported that allantoin accumulated only in nodulated soybean plants and [$^{15}N_2$] supplied to nodulated plants was recovered in allantoin and allantoic acid (24).

The majority (60-80%) of the nitrogen transported in the xylem sap of both nitrogen-fixing cowpeas (19) and soybeans (20) was in the form of ureides. Most of the species transporting ureides were members of the tropical tribe Phaseoleae, of the family Leguminosae, which also includes tribes of nitrogen-fixing plants transporting amides and amino acids, especially glutamine and asparagine (25). Ureide production was correlated with nitrogen fixation, and decreased dramatically in the presence of added nitrate in the growth medium (26).

Low levels of uricase and catalase were associated with the peroxisomal fraction from roots of bean plants (27). Much higher levels of xanthine dehydrogenase, uricase and allantoinase were found in cowpea nodules (28). The addition of allopurinol, an inhibitor of xanthine dehydrogenase, resulted in an accumulation of xanthine and a decrease in ureide formation. Labeled glycine was incorporated into allantoin and allantoic acid in nodule slices (28). A pathway for ureide synthesis through xanthine dehydrogenase was also demonstrated in soybean nodules (29). Ureide metabolism in higher plants has been recently reviewed (30). All of the evidence to date indicates that nitrogen is first assimilated into amino acids which are then incorporated into purines. The purines are degraded to allantoin and allantoic acid, which are exported from the nodule. The pathway of purine synthesis and degradation to allantoic acid appears essentially the same as the pathway previously elucidated in animals. Work is currently underway in several other laboratories to purify the enzymes of purine metabolism from nodules and to isolate the intermediates in this pathway.

The pathway of ureide degradation in soybean leaves is unknown. As described above, low allantoinase activity has been reported in

glyoxysomes from astor bean endosperm (17, 18). Allantoinase activity has been characterized from several other plant tissues (31, 32), including legumes (33, 34). Allantoicase activity was reported from germinating peanuts (35) and leaves of non-nodulated bushbeans (36), and was localized in peroxisomes from the skunk cabbage appendix (37). Allantoinase from skunk cabbage was not peroxisomal, but in the soluble fraction (37). Allantoin induced an increase in glyoxylate in germinating wheat seedlings and labeling studies suggested glycine was a precursor and glyoxylate a hydrolysis product of allantoin (38). Uricase and allantoinase have recently been reported in peroxisomes from mustard cotyledons (39). Much of the literature on this pathway is contradictory, probably due to the very low levels of allantoinase and allantoicase in most plant tissues, and technical difficulties in the assays. These enzymes have not been reported or localized in leaves of nitrogen-fixing plants, in which high levels of activity might be expected.

Statement of the Problem

Since some of the enzymes of purine degradation had been reported in peroxisomes of chicken and fish liver and castor bean endosperm (14, 13, 17) my initial hypothesis was that the enzymes involved in allantoin and allantoic acid metabolism in nitrogen-fixing plants would also be localized within peroxisomes. The presence of the purine degradation pathway in soybean nodules had not been established at the time this project was undertaken, and nothing was known about ureide degradation in soybean leaves. Therefore my task was twofold: first, to identify the enzymes responsible for formation and degradation of ureides in soybean nodules and leaves, and secondly, to determine if these enzymes

were compartmented within peroxisomes. Xanthine dehydrogenase, uricase, and allantoinase activities would be expected in soybean nodules if ureides are produced by the known purine degradation pathway, previously localized in animal peroxisomes (13,14). Allantoinase would catalyze the final reaction taking place in the nodule, since primarily allantoic acid, along with some allantoin, is transported in the xylem sap (20). Allantoinase activity would also be expected in the leaves, since some allantoin is transported. If allantoic acid in soybean leaves is degraded by the same pathway reported in fish peroxisomes (13), glyoxylate and urea would be the products of the allantoicase reaction. Ammonia could be released by urease activity (11). Reactions producing glyoxylate are always found in peroxisomes, where this unstable molecule may be protected from oxidation by H_2O_2 by catalase, and aminotransferases are present to utilize the glyoxylate (1).

Since peroxisomes had never been previously isolated or described from nodules, it was necessary to modify existing methodology for the isolation of nodule peroxisomes. Nodule peroxisomes were isolated by isopycnic sucrose density gradient centrifugation steps, as detailed in Chapter I (40). Xanthine dehydrogenase activity was present in the soluble fraction, uricase and catalase were localized in peroxisomes, and allantoinase activity was found in the microsomal fraction, in contrast to reports from other tissues (13, 17). Marker enzyme data indicated that the microsomes containing allantoinase activity originated from the endoplasmic reticulum.

During the course of this work, we were in communication with Dr. Eldon Newcomb, whose laboratory has been responsible for observation by

electron microscopy of the distribution and ultrastructural complexity of plant peroxisomes and glyoxysomes. Newcomb and Tandon (41) proceeded to examine cytologically the peroxisomal distribution in soybean root nodule cells. They found that enlargement of the peroxisomes and proliferation of smooth endoplasmic reticulum occurs only in the uninfected cells of soybean nodules. This data was consistent with our initial work (40) and further suggested that the uninfected cells might participate in ureide synthesis via the uricase and allantoinase reactions.

In order to investigate this hypothesis, I separated infected and uninfected cell protoplasts on a sucrose step-gradient with a very brief centrifugation, as detailed in Chapter II. Infected protoplasts were much larger, irregular in shape, and more dense than uninfected protoplasts. The peroxisomal enzymes uricase and catalase were primarily associated with the uninfected cell fraction. Allantoinase, previously localized in the endoplasmic reticulum, also had a much greater specific activity in the uninfected cell fraction. Several of the enzymes involved in purine synthesis were present at higher levels in the uninfected cell fraction. These data suggest that at least the latter reactions of ureide formation may take place primarily in the uninfected cells of the soybean nodule.

I have also investigated localization of ureide degradation in soybean leaf cells (Chapter III). Leaf allantoinase was localized within microsomes originating from the endoplasmic reticulum, in agreement with localization in the nodule. No significant allantoicase activity has been found in the leaves, although many different assays were tried. Soybean leaves have also been reported to be very low in urease activity (42).

Germinating seedlings also degrade purines, presumably utilizing ureides stored in the cotyledon (Chapter III). While only traces of uricase were found in seedling peroxisomes, high levels of allantoinase occured in the microsomal fraction. [^{14}C]allantoin was consumed in an *in vivo* assay, indicating allantoicase activity, but this enzyme could not be detected *in vitro*.

Since localization of soybean allantoinase in the endoplasmic reticulum disagreed with previous reports that allantoinase was in peroxisomes from fish liver (13), I investigated the location of enzymes for purine degradation in fish liver (Chapter IV). I found allantoinase only in the soluble fraction, while uricase and allantoicase were peroxisomal. No allantoinase activity was associated with the microsomal fraction. The unstable allantoicase activity in the peroxisomes was increased ten-fold by addition of 10 mM $MgCl_2$.

While many questions remain unanswered, it is the hope of the author that the following chapters will demonstrate the ubiquitous role played by peroxisomes in purine degradation in such diverse tissues as soybean nodules and fish liver and add to our understanding of the importance of compartmentation in metabolic regulation in all living cells.

CHAPTER I

PEROXISOMES FROM SOYBEAN NODULES

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Localization of Enzymes of Ureide Biosynthesis in Peroxisomes and Microsomes of Nodules[1]

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ABSTRACT

The intracellular location of enzymes involved in the synthesis of the ureides, allantoin and allantoic acid, was investigated in nodules of *Glycine max* L. Merr. Cellular organelles were separated on isopycnic sucrose density gradients. Xanthine dehydrogenase activity (270 nanomoles per min per gram fresh weight) was totally soluble, whereas approximately 15% of the total uricase and catalase activities (1 and 2000 micromoles per minute per gram fresh weight, respectively) was in the fraction containing intact peroxisomes. Allantoinase activity (680 nanomoles per minute per gram fresh weight) was associated with the microsomal fraction, which apparently originates from the endoplasmic reticulum.

The ureides, allantoin and allantoic acid, are the predominant form of nitrogen transported in the xylem of soybean and cowpea plants growing symbiotically (10, 16). The synthesis of allantoic acid presumably occurs via the degradation of purines (1, 24). After differential centrifugation of extracts from cowpea or soybean nodules, the enzymes of purine catabolism were found in the soluble fraction (1, 24). These results do not rule out the possibility that these enzymes are located in fragile organelles, such as peroxisomes. This possibility is supported by results after careful fractionation of nodule extracts by differential centrifugation (19).

One enzyme in the purine degradation pathway, uricase, is normally found in peroxisomes, along with catalase, which degrades the H_2O_2 produced by uricase. Small amounts of uricase have been reported to be present in glyoxysomes of germinating fatty seeds (23) and in microbodies from potato tubers (18). Traces of uricase are also present in peroxisomes from other plant tissues (12, 13). In all of these reports, uricase was easily solubilized and did not appear to be part of the crystalline core of the peroxisome. Xanthine dehydrogenase is generally found in the cytosolic fraction, although reportedly it is present in peroxisomes from avian livers (20). Allantoinase and allantoicase have been reported to be present in peroxisomes from amphibian (20) and fish (17) livers. Approximately one-half of the allantoinase activity in castor bean endosperm was associated with glyoxysomes and the remainder was in the proplastid region. Eighty percent of the total activity was lost on the gradient (23). In another report on castor bean endosperm allantoinase, most of the activity was found in fractions of density 1.21 g/cc or greater. In this case, the allantoinase activity, which was reported only as *A* units, appeared to be extremely low and the location of other organelles was not adequately characterized (22). At least part of the pathway of purine catabolism has been considered to be associated with animal peroxisomes and possibly with plant microbodies. This project was initiated to examine the organelle distribution of the enzymes associated with allantoin and allantoic acid formation in nodules, which form these compounds from recently fixed nitrogen for transport to the leaves and pods.

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MATERIALS AND METHODS

Seeds of *Glycine max* L. Merr. cv. Amsoy 71 were inoculated with *Rhizobium japonicum* strain 311b 110 obtained from D. Weber, United States Department of Agriculture, Beltsville, MD. Plants were grown in a growth chamber in perlite and watered daily with nitrogen-free nutrient solution. Nodules (1.2 g) were harvested from 30-day-old plants and very gently broken in a mortar containing 10 ml medium (8) containing 0.4 M sucrose, 0.1 M Tricine (pH 7.8), 10 mM DTT, 10 mM KCl, 1 mM $MgCl_2$, and 10 mM EDTA, plus 50 mg fatty acid-free BSA and 100 mg soluble PVP. The entire extract was squeezed through six layers of cheesecloth and applied to a step gradient of 3 ml 2.3 M sucrose, 5 ml 1.9 M sucrose, 10 ml 1.8 M sucrose, 10 ml 1.75 M sucrose, 10 ml 1.7 M sucrose, 6 ml 1.5 M sucrose, and 6 ml 1.3 M sucrose. To obtain a better separation of microsomes, a gradient of 3 ml 2.3 M sucrose, 5 ml 1.9 M sucrose, 7 ml 1.8 M sucrose, 7 ml 1.75 M sucrose, 7 ml 1.7 M sucrose, 7 ml 1.5 M sucrose, 6 ml 1.3 M sucrose, 4 ml 1.0 M sucrose, and 4 ml 0.83 M sucrose, was used. All sucrose solutions were prepared in 0.1 M Tricine (pH 7.8).

Gradients were centrifuged in a Beckman SW 25.2 swinging bucket rotor at 4 C in a Beckman L-2 ultracentrifuge. The speed was slowly accelerated by holding for 15 min each at 5,000, 10,000, 15,000, and 20,000 rpm, and then run for 5 h at 25,000 rpm (106,900*g*). Fractions from 2 to 5 ml were collected from the top of the gradient using an ISCO model 185 Density Gradient Fractionator.

Catalase activity was determined by the decrease in *A* at 240 nm (14). Uricase activity was determined by the decrease in *A* at 293 nm in a 1-ml assay mixture containing 0.1 mM uric acid in 0.1 M 2-*N*-cyclohexylaminoethanesulfonate (Sigma) buffer at pH 10 (19). Nodular uricase was inhibited by borate buffer and Triton X-100. NADH-Cyt *c* reductase activity was measured by the NADH-dependent increase in *A* at 550 nm (4), using an extinction coefficient of 21 mM^{-1} cm^{-1} (15). Cyt *c* oxidase activity was determined by the decrease in *A* of reduced Cyt *c* at 550 nm (21), after the extract alone was incubated with 20 μl 1% digitonin for 1 min before addition of buffer and substrate. Triosephosphate isomerase activity was determined by coupling to α-glycerophosphate dehydrogenase (EC 1.1.99.5) (2). Potassium-stimulated

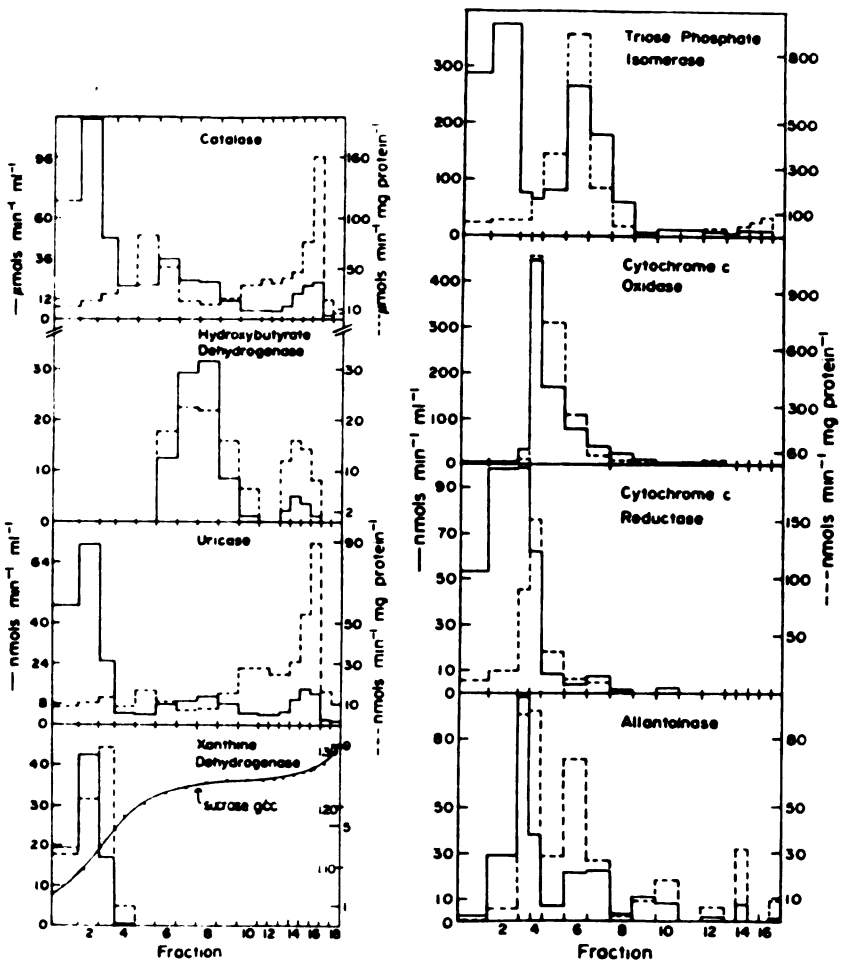

FIG. 1. Distribution of organelle marker enzyme activities (——) and specific activities (- - -) on a sucrose gradient after centrifugation for 6 h of a nodule homogenate which contained BSA in the grinding media and was applied directly to the gradient. Units are nmol min⁻¹ ml⁻¹ except catalase which is in μmol min⁻¹ ml⁻¹. Peroxisomes banded at a density of 1.25 g/cc.

ATPase was assayed by measuring the Pi released (11). IDPase[2] activity was measured 48 h after extraction of tissue by determining Pi released from IDP (4).

The xanthine dehydrogenase assay mixture contained 5 μmol NAD, extract, and 0.1 M Tricine (pH 8.4), in a final volume of 1 ml. The endogenous rate at 340 nm was measured and the reaction was initiated with the addition of 0.25 μmol xanthine. Hydroxybutyrate dehydrogenase activity was measured after incubation of extract with 50 μl 10% Triton X-100 for 2 min, and then addition of 5 μmol NAD and 0.1 M Tricine (pH 7.8) to a volume of 1.0 ml. The increase in A at 340 nm was measured. The reaction was initiated by the addition of 50 μmol β-DL-hydroxybutyrate.

Allantoinase activity was measured by formation of the product, allantoic acid. Allantoic acid was determined by boiling in dilute acid and measuring the diphenylformazan derivative of the glyoxylate produced (26). A substrate concentration of 15 mM allantoin in 20 mM Tricine (pH 7.8) was used in the assay performed on gradient fractions. It was necessary to use a concentration slightly lower than the K_m for allantoin (approximately 19 mM, data not shown), because of the low solubility of allantoin and the high background caused by nonenzymic breakdown of substrate. A 30 mM allantoin reagent was used to estimate total enzyme activity in the homogenate. The assays were stopped after 20 min with 1 ml 0.15 M HCl. Sucrose and other components of the enzyme sample reacted with phenylhydrazine in this assay to give an orange color, which in some cases obscured the diphenylformazan product at 520 nm. This artifact could be minimized by boiling the samples and then briefly chilling in ice before adding phenylhydrazine. The use of ultrapure density gradient grade sucrose (Mann Research Laboratories) also decreased the interference with the assay. Controls were run for nonenzymic breakdown of substrate, as well as for the presence of product at zero time in the extract.

Protein was determined by a modified Lowry procedure (3). Phosphate was determined by the method of Chen *et al.* (5).

RESULTS AND DISCUSSION

Catalase and uricase, enzymes found in peroxisomes of animal and plant tissues (12, 17), were present at high levels in soybean

[2] Abbreviations: IDPase, inosine diphosphatase

Table I. *Separation of Enzymatic Activities in Fractions Containing Peroxisomes and Bacteria*

Gradient fractions from 1.24–1.27 g/cc sucrose, containing a mixture of peroxisomes and bacteria, were combined, diluted with two parts buffer, vortexed 1 min, and centrifuged 1 h at 145,000*g*. Solubilized peroxisomal enzymes appeared in the supernatant, while the pellet contained the bacteria.

	Initial Combined Fractions	After Breakage and Recentrifugation	
		Supernatant	Pellet
Catalase			
nmol/min	56	53	0.1
nmol/min·mg protein	160	280	0.25
Uricase			
nmol/min	36	32	0.05
nmol/min·mg protein	89	164	0.12
Hydroxybutyrate dehydrogenase			
nmol/min	14	0	8.1
nmol/min·mg protein	16	0	15

nodules. Nodules contained approximately 2000 μmol min^{-1} g fresh weight^{-1} catalase activity and 1 μmol min^{-1} g fresh weight^{-1} uricase activity. This level of uricase is at least 10 times that detected in other plant tissues (12, 13, 18, 23) including potato tubers and glyoxysomes, and is comparable to that found in rat liver on a protein basis. In initial attempts at isolating peroxisomes all of the catalase and uricase was found in the soluble fraction at the top of the gradient or in the bacteroid fraction. Various methods of chopping the tissue, the use of different grinding media and gradients, and low speed precentrifugation were tried in order to isolate a significant peroxisomal fraction. Separation of peroxisomes from the large numbers of mature bacteroids and vegetative cells presented a major problem, because both peroxisomes and bacteria have nearly the same density. To isolate a peroxisomal fraction very gentle breakage of the nodules was necessary, and only young nodules were used. It was necessary to increase the centrifugation time to 6 h and broaden the sucrose gradient in the denser region in order to separate peroxisomes from vegetative cells (Fig. 1). Nodule peroxisomes appeared to be extremely fragile, as judged from the large amounts of soluble catalase and uricase with even very gentle homogenization. Tandon and Newcomb (manuscript in preparation) are also reporting, based on observations by electron microscopy, that the peroxisomes in nodules appear broken and degenerated, particularly in older tissue, and sometimes do not have a bounding membrane.

Results of enzyme assays after fractionation of organelles on a sucrose density gradient are presented in Figure 1. In the upper fractions, which represent soluble enzymes, some sedimentation occured into fraction 2. Xanthine dehydrogenase activity appeared only in the soluble portion of the gradient. A large fraction of the catalase and uricase activities, presumably from broken peroxisomes, was also at the top of the gradient. Intact peroxisomes, localized by catalase and uricase activities, appeared at a sucrose density of 1.25 g/cc and contained approximately 15% of the total catalase and uricase activity. Allantoinase activity coincided with the microsomal marker, Cyt *c* reductase (Figs. 1 and 2). No activity of glucose-6-phosphatase, another microsomal marker, could be detected.

Two peaks of bacterial origin were observed using the marker enzyme, hydroxybutyrate dehydrogenase. The band of lower density was presumed to be mature bacteroids, and the band of higher density, which overlapped the peroxisomal band, to be vegetative cells (6). Other marker enzymes used were Cyt *c* oxidase for mitochondria and triosephosphate isomerase for proplastids (Fig. 1).

Specific activities of the marker enzymes are shown as a dashed line in Figure 1. Specific activities for soluble enzymes in the upper three or four fractions were low because of the addition of BSA to the grinding media, and as a result, the specific activity peaks of the microsomal marker enzyme, Cyt *c* reductase, and allantoinase were shifted toward higher densities of sucrose than the plotted peaks for total activity. The specific activities of catalase in the peroxisomes was 160 μmol min^{-1} mg protein^{-1} and for uricase it was 89 nmol min^{-1} mg protein^{-1}.

In order to assay hydroxybutyrate dehydrogenase, it was necessary to break the bacteroids by sonication or treatment with Triton X-100. No activity could be measured prior to such treatment. Catalase and uricase activity could be measured without sonication or treatment with detergent, and actually decreased if sonicated (data not shown). This suggested that these enzyme activities in fractions of density 1.24 to 1.27 g/cc were derived from two different organelles.

To test the hypothesis that hydroxybutyrate dehydrogenase activity in the peroxisomal band was due to contaminating bacteria, fractions at approximately 1.24 to 1.27 g/cc sucrose were combined, diluted with two parts buffer, and vortexed to break the fragile peroxisomes. The sample was then centrifuged 1 h at 145,000*g* in a Beckman TY 65 rotor, and the pellet and soluble fraction were assayed separately. The pellet was washed with 0.5 ml buffer, and resuspended in 1.0 ml. Catalase and uricase activity were in the soluble fraction (Table I), while the hydroxybutyrate dehydrogenase activity remained in the pelleted bacteroids. The specific activity of catalase and uricase increased greatly in the soluble fraction, due to the removal of the large amount of bacterial protein.

To examine the organelle location of allantoinase further, the sucrose density gradient was broadened slightly to give better separation in the area of the microsomes (Fig. 2). DTT and soluble PVP, which were found to inhibit allantoinase, were omitted in this gradient. Allantoinase cosedimented with Cyt *c* reductase, a microsomal marker. As before, specific activities in the upper fractions were low, due to BSA in the grinding media. Very few units of allantoinase were in the top fractions, and this suggests that the enzyme may be membrane-bound as it was not solubilized upon breakage of the ER. The activity of IDPase, a marker enzyme for Golgi apparatus in plants (4), was mainly in the soluble fraction. The potassium-stimulated ATPase, a marker enzyme for the plasma membrane in plants (11), was also in the soluble fraction. Thus, it appeared that the allantoinase activity in the microsomes did not originate from either Golgi or plasma membranes. Whereas allantoinase and Cyt *c* reductase cosediment, it is probable that allantoinase is located in the ER. The possibility still exists that the microsomes to which allantoinase is bound arise from the broken fragments of peroxisomal membranes or from some other source.

CONCLUSIONS

Based on the distribution of the enzymes of purine catabolism, it is suggested that the intermediates in the ureide pathway are metabolized in several locations in the cell (Fig. 3). Nitrogenase is contained within the bacteroids, which release ammonia to the host cell. We have pictured glutamate synthase and glutamine synthetase in the proplastids, inasmuch as these enzyme activities have been localized in leaf chloroplasts (25). Amino acids are then used in the synthesis of purines (1), which are degraded to xanthine (25) in the cytosol. Xanthine appears to be converted to uric acid in the cytosol by xanthine dehydrogenase. Uric acid is oxidized to allantoin in the peroxisomes by uricase, producing H_2O_2, which is degraded by catalase, also located in the peroxisomes. Allantoin then appears in the xylem sap (16) or is hydrolyzed to allantoic acid by allantoinase in the ER, and allantoic acid secreted into

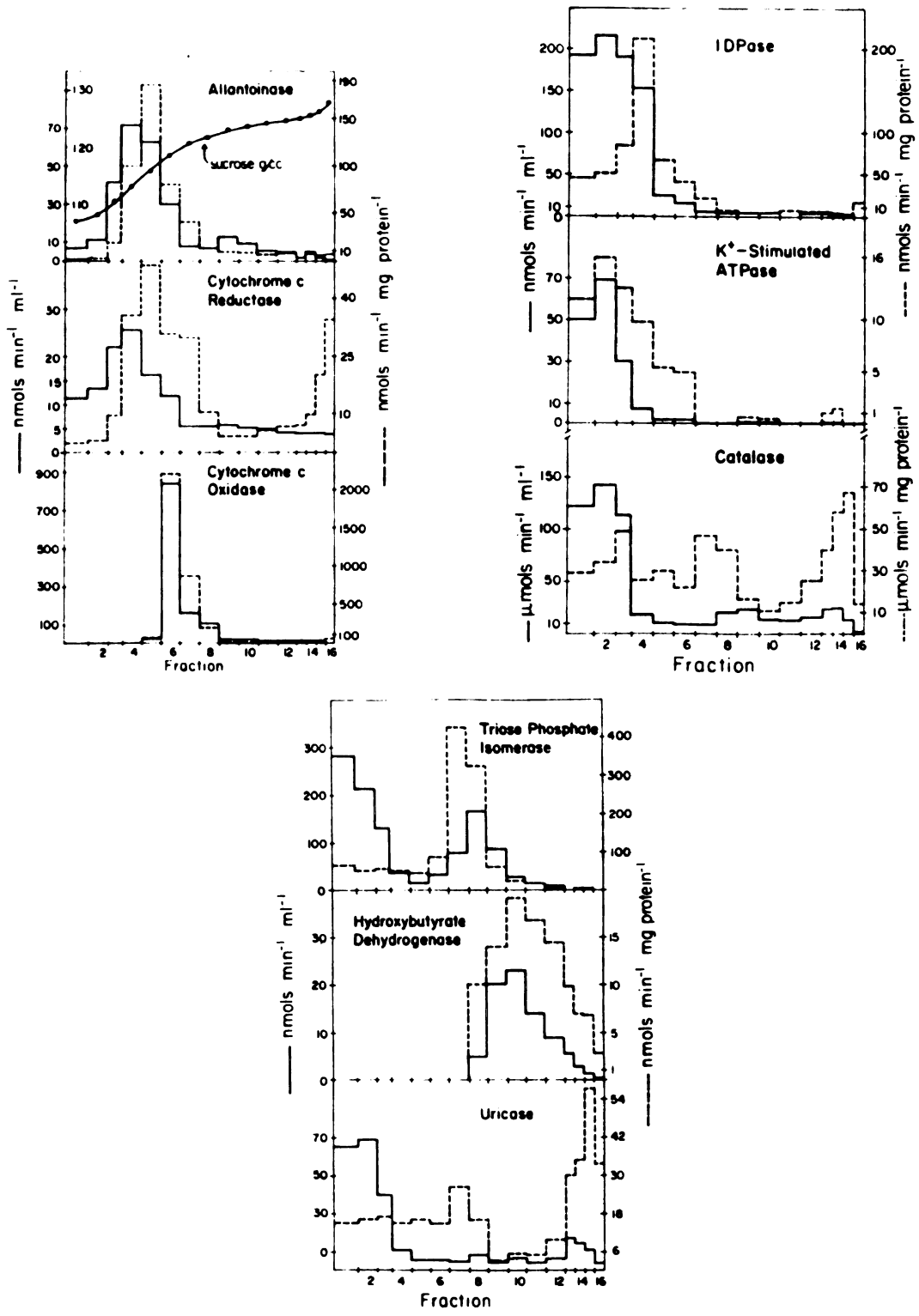

FIG. 2 Distribution of organelle marker enzyme activities (——) and specific activities (- - -) from a nodule homogenate which contained BSA in the grinding media and was applied directly to a broadened sucrose gradient, and centrifuged for 6 h. Units are nmol min^{-1} ml^{-1} except catalase which is in μmol min^{-1} ml^{-1}. Note that microsomal enzymes banded at a density of 1.18 g/cc.

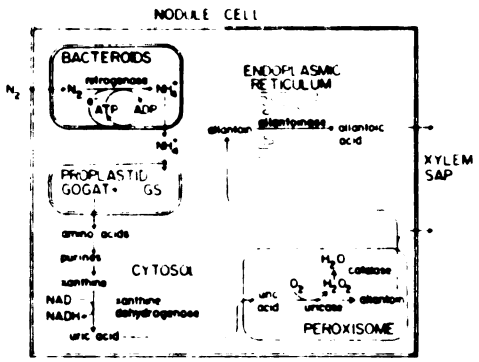

FIG. 3. Schematic representation of the proposed intracellular location of the enzymes of purine degradation. GOGAT, glutamate synthase; GS, glutamine synthetase.

the xylem, where both ureides are translocated to the aerial parts of the plant. In such a model, the ER may be involved in the release of the negatively charged allantoic acid. This hypothesis is consistent with the suggestion that the ER may play a role in secretion of both large and small molecules and in regulation (7). The model requires also that uric acid and allantoin must cross the single peroxisomal membrane by diffusion.

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Additional Enzyme Activities in Nodule Peroxisomes

Nodule peroxisomes were also analyzed for activities of enzymes found in leaf peroxisomes or glyoxysomes. A complete glyoxylate cycle is present only in germinating seeds (8). An incomplete cycle, lacking isocitrate lyase, is present in resting seeds (58). No isocitrate lyase activity was found in nodule peroxisomes, and only traces of malate synthetase were present. Glycolate oxidase was not present in nodule peroxisomes. A low level of aspartate aminotransferase was found in the peroxisomal fraction, but the majority of this enzyme was in the plastid and soluble fractions (Table 2). Hydroxypyruvate reductase activity was absent from the peroxisomes, although some soluble activity was present. This activity may be attributed to lactate dehydrogenase, which was also present at about the same level in the soluble fraction. Only traces of activity were found for the glyoxylate:glutamate and glyoxylate:serine aminotransferases, normally found at high levels in leaf peroxisomes. Thus it appears that nodule peroxisomes do not contain the enzymes found in leaf peroxisomes or glyoxysomes, but are specialized only to perform the uricase and catalase reactions, which are essential for the production of allantoin in soybean nodules.

Table 2

Additional Enzyme Activities

in Nodule Peroxisomes

Enzyme	Soluble		Peroxisomal	
	Activity (units) nmol $min^{-1}ml^{-1}$	Specific Activity units mg $protein^{-1}$	Activity (units) nmol $min^{-1}ml^{-1}$	Specific Activity units mg $protein^{-1}$
Isocitrate lyase	0		0	
Malate synthetase	trace		trace	
Glycolate oxidase	0		0	
Aspartate aminotransferase	890	120	100	220
Hydroxypyruvate reductase	92	12	0.7	1.6
Lactate dehydrogenase	56	7.4	0	
Glyoxylate:glutamate aminotransferase	trace		trace	
Glyoxylate:serine aminotransferase	trace		trace	

CHAPTER II

UREIDE METABOLISM IN INFECTED AND UNINFECTED NODULE CELLS

INTRODUCTION

Allantoin and allantoic acid, the ureides, are the major nitrogenous compounds transported in the xylem sap of soybeans (20). Labeling studies have indicated that the high levels of purines synthesized in the nodule are subsequently degraded to ureides (28). The role of both cellular and sub-cellular compartmentation in this process has been implicated in several recent reports. Uricase and catalase were localized in peroxisomes, allantoinase in the endoplasmic reticulum, and xanthine dehydrogenase in the cytosol by fractionation of a total nodule tissue extract on sucrose density gradients (40). By means of electron microscopy, a marked enlargement of peroxisomes and proliferation of smooth endoplasmic reticulum during nodule development was observed to occur only in the uninfected cells of nodules, indicating an important role of these cells in ureide production (41). In further cell fractionation studies, several enzymes involved in ammonia assimilation into amino acids and purine synthesis were localized in the plastid (Appendix I, 59). These included asparagine synthetase, phosphoribosyl amidotransferase, phosphoglycerate dehydrogenase, serine hydroxymethylase, methylene tetrahydrofolate dehydrogenase, one isozyme of aspartate aminotransferase, glutamate synthase, and triosephosphate isomerase.

Separation of infected and uninfected nodule cells is necessary to determine the distribution of enzymes and organelles involved in ureide production. I have separated protoplasts on a sucrose step-gradient and assayed the uninfected and infected cell fractions for enzymes involved in purine synthesis and degradation.

MATERIALS AND METHODS

Seeds of Glycine max were inoculated with Rhizobium japonicum strain 3I1b 110 obtained from D. Weber, United States Department of Agriculture, Beltsville, MD. Plants were grown in a growth chamber in perlite and watered daily with nitrogen-free nutrient solution. For protoplast isolation, 6 g of nodules from 50 day-old plants were finely sliced with a razor blade in a petri dish containing about 5 ml of 1-B5 tissue culture medium of Gamborg (60). This medium was removed with a pipet, and the tissue was rinsed four times with fresh 1-B5 medium to remove broken cell contents. Then 11 ml of 1-B5 medium and an enzyme solution containing 200 mg Cellulysin (Calbiochem), 100 mg Hemicellulase (Sigma), and 0.2 ml Pectinase (Sigma), 1 g sorbitol, and 9 ml H_2O were added. The dish was shaken at 25 rpm for 1.5 to 2.5 h at 25°C. The brei was passed through 100 um nylon mesh. Approximately 16 ml of the protoplast suspension were very gently layered onto the following step gradient, prepared immediately before use, in a 30 ml nitrocellulose tube: 3 ml of 60% sucrose, 7 ml of 40% sucrose, and 10 ml of B5 medium (60). All sucrose solutions were prepared in B5 medium. The gradient was centrifuged at low speed (about 30 x g) in a clinical swinging bucket centrifuge for 3 to 5 minutes, when two layers of cells at the interfaces could be seen. One ml fractions were collected from the top of the gradient using an ISCO model 185 Density Gradient Fractionator. It was not possible to perform cell counts due to the low yield of purified protoplasts, nor could bacteroids be separated from cellular membranes of the infected cell fraction.

Uricase, allantoinase and hydroxybutyrate dehydrogenase were assayed as previously reported (Chapter I, 40). Catalase was determined by the decrease in absorbance at 240 nm (53). Triosephosphate isomerase was determined by coupling to α-glycerophosphate dehydrogenase (43). Aspartate aminotransferase was assayed by coupling to malate dehydrogenase (61). Phosphoribosyl amidotransferase was assayed by 5-phospho-α-D-ribose 1-pyrophosphate (PRPP)-dependent deamidation of [^{14}C]glutamine (62), which was separated from labeled glutamate by ion exchange (63). Phosphoglycerate dehydrogenase was assayed by phosphohydroxypyruvate-dependent oxidation of NADH at 340 nm (59). The assay mixture for glucose6-phosphate dehydrogenase contained 2 mM glucose-6-phosphate, 0.2 mM NADP, and 20 mM tricine, pH 7.8. The increase in absorbance at 340 nm was measured. The assay for 6-phosphogluconate dehydrogenase was identical, except for substitution of 2 mM 6-phosphogluconate for glucose-6phosphate. Lactate dehydrogenase was assayed at 340 nm with 0.15 mM NADH, 2 mM pyruvate or hydroxypyruvate, and 50 mM phosphate buffer, pH 7.5. The assay mixture for malate dehydrogenase contained 0.2 mM NADH, 3.3 mM oxaloacetate, and 20 mM tricine, pH 7.8. Decrease in absorbance at 340 nm was measured. Native polyacrylamide gel electrophoresis was performed according to Laemmli (64), omitting sodium dodecyl sulfate. the gels were stained for aspartate aminotransferase activity with Fast Violet B salt (Sigma) (65). Protein was determined by a modified Lowry procedure (44).

RESULTS AND DISCUSSION

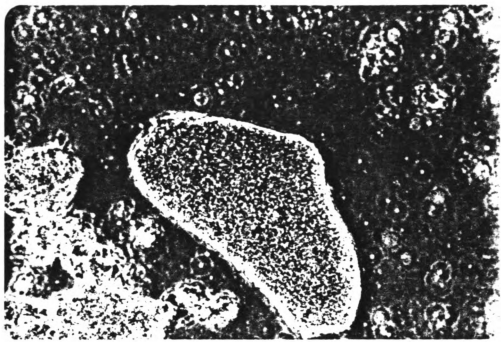
Although protoplasts have been previously isolated from leguminous nodules (66, 67), only infected cells were obtained. As illustrated in Figure 5, the uninfected protoplasts we isolated were much smaller than the infected protoplasts and were spherical, while the infected protoplasts were irregular in shape and had a granular surface. Staining with Calcofluor white indicated no cell wall material remained. The uninfected cells did not come from the cortex, since control experiments using only infected tissue yielded both uninfected and infected protoplasts, and no protoplasts were obtained from cortex tissue in 3 hours digestion. The uninfected protoplasts proved to be more fragile and were often obtained in lower yield than the infected protoplasts (Table 3). The infected protoplasts had a very high density, probably due to the large number of bacteroids per cell, and rapidly pelleted through 50% sucrose even at low centrifugal force. Attempts to separate the two types of protoplasts by flotation were unsuccessful, and the density of the dextran gradients of Edwards et al. (68) were not high enough to separate the infected protoplasts. The sucrose step-gradient specified above was designed to sediment the uninfected protoplasts at the upper interface, and the infected protoplasts at the lower interface. It was essential to minimize handling of the protoplasts, since the uninfected cells were easily broken by contact with the larger infected cells and the high sucrose concentration necessary for separation. Yields of each protoplast type ranged from 0.5 to 3.0 mg protein, depending on the amount of tissue used and incubation time. Longer periods of incubation with the digestive enzymes yielded fewer uninfected cells but more

Figure 5. Protoplasts of infected and uninfected soybean nodule cells. Light micrographs were of a crude protoplast suspension photographed before separation on the step-gradient. Background debris from broken cells was removed in the upper layer of the gradient. Magnification: 25X.

a) infected cell. Size ranged from 100-200 um in diameter.

b) uninfected cell. Size ranged from 20 to 50 um in diameter.

a



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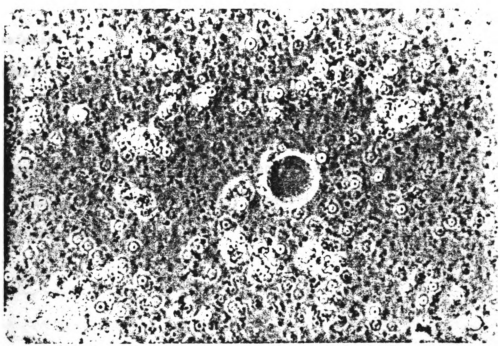

Table 3

Total Enzyme Units and Specific Activity from a Representative Step-Gradient

	Uninfected Cell Fraction		Infected Cell Fraction	
	nmol min^{-1}	Specific Activity	nmol min^{-1}	Specific Activity
Uricase	20	51	4.5	5.5
OH-butyrate dehydrogenase	0.2	0.6	3.5	4.3
Phosphoglycerate dehydrogenase	25	64	20	24
Aspartate aminotransferase	20	51	18	22
Protein (mg/ml)	0.39		0.82	

Protoplasts were separated on a sucrose step-gradient as in Materials and Methods. All fractions were 1.0 ml. Specific activity units are nmol min^{-1} mg $protein^{-1}$.

infected cells, and often resulted in higher levels of cross contamination. Only by using the 1-B5 and B5 media of Gamborg (60) were substantial quantities of uninfected protoplasts obtained. More uninfected protoplasts were obtained from nodules of 40-50 day old plants than from nodules of younger plants.

Data from a representative separation of infected and uninfected protoplasts is presented in Table 3. Uricase was the marker enzyme for peroxisomes (40) and hydroxybutyrate dehydrogenase was the bacteroid marker enzyme. The lower protoplast fraction at the 60% sucrose interface contained most of the hydroxybutyrate dehydrogenase activity, and was therefore designated the infected cell fraction. The upper protoplast fraction at the 40% sucrose interface was very low in hydroxybutyrate dehydrogenase activity and was designated the uninfected cell fraction. Uricase activity was primarily found in the uninfected cell fraction. Data are also shown for phosphoglycerate dehydrogenase and aspartate aminotransferase, two of the enzymes localized in plastids which are probably involved in purine synthesis (Appendix, 59). The specific activities of these two enzymes were 2 to 3 times higher in the uninfected cell fraction, but about half of the total activity was also present in the uninfected cell fraction, but about half of the total activity was also present in the infected cell fraction. On the basis of total mg protein, the yield of uninfected protoplasts was about half that of infected cell protoplasts. Triton-X-100 was present only in the assay mixture for hydroxybutyrate dehydrogenase, in order to break the bacteroid membranes (40). Addition of low concentration of detergent (.01%) did not increase any of the other enzyme activities, indicating cellular membranes were probably broken during dilution into the assay mixture, and were not a barrier to enzyme activity.

Average specific activities from 25 different step-gradients are presented in Table 4. Uricase and catalase, the peroxisomal enzymes (40), were predominantly in the uninfected cell fraction. Allantoinase, located in the endoplasmic reticulum (40), was also mostly in the uninfected cell fraction. These data confirm the hypothesis of Newcomb and Tandon (41) that peroxisomes and the endoplasmic reticulum in the uninfected cells contain the enzymes which catalyze the final steps in ureide formation.

Average specific activities for several of the metabolic enzymes which are involved in purine biosynthesis are given in Table 5. Since none of these assays contained detergent, it may be assumed that the bacteroids made no contribution to the activities as measured. Phosphoglycerate dehydrogenase is a plastid enzyme, probably involved in synthesis of serine, which is required for purine biosynthesis (59). The specific activity of phosphoglycerate dehydrogenase was twice as high in the uninfected cell fraction as in the infected cell fraction.

Specific activity of aspartate aminotransferase (Table 5) was 2-3 times higher in the uninfected cell fraction. One isozyme of aspartate aminotransferase in the soybean nodule has been localized in the plastid (59), and aspartate is required for purine synthesis. The presence of different isozymes of aspartate aminotransferase in peroxisomes, mitochondria and chloroplasts of leaves has been previously reported (65).

Protoplast fractions were subjected to native polyacrylamide gel electrophoreis and stained for aspartate aminotransferase activity (Figure 6). Band 1 is the soluble isozyme, and band 2 is the plastid isozyme (59). The uninfected cell fraction was applied to gel A,

Table 4

Specific Activity of Enzymes Involved in Ureide Formation

	Uninfected Cell Fraction	Infected Cell Fraction
	nmol mg^{-1} mg $protein^{-1}$	
Uricase	65	4.1
Catalase	35×10^3	5.0×10^3
Allantoinase	9.0	3.6
OH-butyrate dehydrogenase	1.5	6.0

Triton X-100 was present only in the assay for hydroxybutyrate dehydrogenase.

Table 5

Specific Activity of Enzymes Involved in Purine Synthesis and Energy Metabolism

	Uninfected Cell Fraction	Infected Cell Fraction
	nmol min^{-1} mg $protein^{-1}$	
Phosphoglycerate dehydrogenase	58	27
Aspartate Aminotransferase	79	30
6-Phosphogluconate Dehydrogenase	4.4	1.1
Glucose-6-Phosphate Dehydrogenase	3.7	1.1
Triose-P Isomerase	150	140
Malate Dehydrogenase	7.2	3.5
Lactate Dehydrogenase	7.0	5.4

Triton X-100 was not present during the assay.

Figure 6. Isozymes of aspartate aminotransferase. Proteins were separated by native polyacrylamide gel electrophoresis and stained for aspartate aminotransferase activity with Fast Violet B. Band 1 is the soluble isozyme; band 2 is the plastid isozyme (59). A = uninfected cell fraction, B = infected cell fraction.

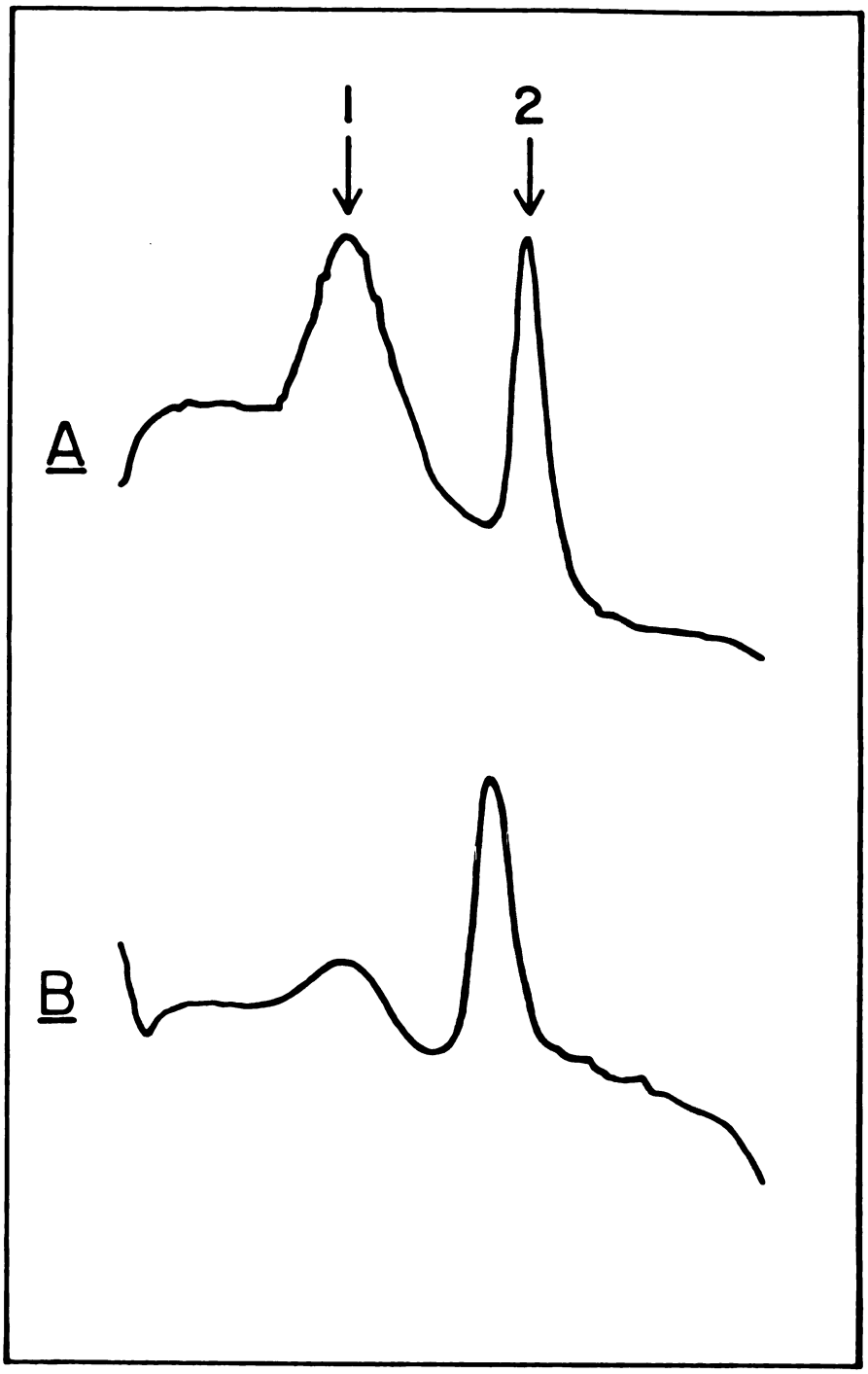

Figure 6. Isozymes of Aspartate Aminotransferase

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which contained much more activity in Band 1, the soluble isozyme. The infected cell fraction was applied to gel B. Both gels showed essentially equal amounts of the plastid isozyme (band 2). The amount of activity of the soluble isozyme in the infected cell fraction (B) was due at least in part to contamination by uninfected cells (indicated by uricase activity).

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are enzymes of the oxidative pentose phosphate pathway, one product of which is ribulose-5-phosphate, which is converted to ribose-5-phosphate by ribose-phosphate isomerase (69). Ribose-5-phosphate is one of the substrates of 5-phospho-α-D-ribose 1-pyrophosphate (PRPP) synthetase, in the first step leading to purine biosynthesis. Therefore, high levels of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase would be expected in cells synthesizing large amounts of purines. The specific activity of both of these enzymes in the uninfected cell fraction was about four times the specific activity in the infected cell fraction (Table 5). Starch is the major source of glucose-6-phosphate in most plant cells (70). I have noted starch granuales in the plastids of only the uninfected cells in the electron micrographs of Newcomb and Tandon (41).

Triosephosphate isomerase activity is present in both the cytosol and plastid fractions (59). The specific activity of this enzyme was essentially equal in both uninfected and infected cell fractions. The specific activity of malate dehydrogenase in the uninfected cell fraction was about twice that in the infected cell fraction (Table 5). While nodule peroxisomes contained no hydroxypyruvate dehydrogenase, lactate dehydrogenase was present in the cytosol, and utilized

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pyruvate, hydroxypyruvate, or glyoxylate as a substrate (Chapter I). The specific activity of lactate dehydrogenase was only slightly higher in the uninfected cell fraction (Table 5).

Results of assays for phosphoribosyl amidotransferase activity were inconclusive. Both uninfected and infected cell fractions contained very low levels of this enzyme activity. While this enzyme has been measured in nodule extracts, the specific activity was low compared with uricase (71), for example, and one would not expect to detect activity in the low yields of pure protoplasts obtained in our experiments. PRPP synthetase was also not detectable. It will probably only be possible to localize these enzymes in protoplasts by labeling studies. Phosphoribosyl amidotransferase has been localized intracellularly in the plastid (59).

SUMMARY

Several intracellular compartments of nodule cells participate in purine synthesis and degradation to ureides, including plastids, peroxisomes, the endoplasmic reticulum, and the cytosol (59, 40). Most of the activity of the peroxisomal enzymes uricase and catalase was associated with the uninfected cell fraction. Allantoinase, which has been localized in the endoplasmic reticulum (40), also had a much greater specific activity in the uninfected cell fraction. All of these data support the previous report (41), based on electron micrographs, that peroxisomes are found predominantly in the uninfected cells, where smooth endoplasmic reticulum also proliferates.

Purine synthesis has been localized in the plastids (59), which are observed in both cell types in the soybean nodule (41). Purine synthesis might therefore occur primarily in only infected cells, only in uninfected cells, or in both cell types. Several of the enzymes whose products are required for purine synthesis, inlcuding phosphoglycerate dehydrogenase, aspartate aminotransferase, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase, were present at much higher levels in uninfected cells, and the soluble isozyme of aspartate aminotransferase was predominantly found in the uninfected cell fraction. However, the possibility of purine synthesis also occurring in the infected cells cannot be excluded on the basis of enzyme distribution alone. If purine synthesis does occur primarily in the uninfected cells, amino acids are probably transported from the infected cells to the uninfected. But if purine synthesis occurs in the infected cells, a purine intermediate might be transported. These and other questions may be resolved by labeling studies using purified protoplasts.

CHAPTER III

METABOLISM OF UREIDES IN SOYBEAN LEAVES AND SEEDLINGS

Allantoin and allantoic acid, the major transport forms of nitrogen in soybean xylem sap, must be hydrolyzed in the leaves in order for the nitrogen to be useful in the growth of the shoot. Our knowledge concerning allantoinase, allantoicase, and urease, the enzymes likely to be responsible for the hydrolysis of the ureides in soybean leaves, is fragmentary, as indicated in the introduction.

METHODS

1. Sorbitol gradients of leaf organelles.

Soybean plants were grown as described in Chapter I. Ninety grams of leaves were homogenized in a Waring blendor with 450 ml of 15% sorbitol in 0.1 M Tricine (pH 7.8) with 40 g buffer-saturated insoluble polyvinylpolypyrrolidone. To remove debri, the homogenate was filtered through four layers of cheesecloth and one layer Miracloth, then centrifuged at 300 g for 15 minutes. The supernatent was centrifuged at 20,000 g for 30 minutes. The pellet was resuspended in 10 ml of the above medium and layered on a step gradient of 10 ml of 85% (w/v) sorbitol, 10 ml of 70% sorbitol, 10 ml of 60% sorbitol, 10 ml of 50% sorbitol, and 10 ml of 30% sorbitol. All sorbitol solutions were prepared in 0.1 M Tricine (pH 7.8). The gradient was slowly accelerated for one hour to 106,900 g and centrifuged for 4 hours at this speed.

2. Sucrose gradients of leaf organelles.

Soybean leaves (100 g) were homogenized in a Waring blendor with 50 g buffer-saturated insoluble PVP and 475 ml of medium containing 15% sorbitol, 0.1 M tricine (pH 7.8), 10 mM KCl, 1 mM $MgCl_2$, and 10 mM

EDTA. The homogenate was centrifuged as above. The 20,000 g pellet was applied to the microsomal sucrose gradient given in Chapter I, and centrifuged at 106,900 g as above.

3. Sucrose gradients of seedling organelles

Eighty g of shoots from 6 day old seedlings were homogenized in 175 ml of the medium used for sucrose gradients (above) with 38 g buffer-saturated insoluble polyvinylpolypyrralidone. The homogenate was filtered and centrifuged as for the above leaf gradients. The 20,000 g pellet was applied to the microsomal sucrose gradient given in Chapter I, and centrifuged at 106,900 g as above.

4. Enzyme assays

Marker enzymes were assayed as in Chapter I. Chlorophyll was determined in 80% acetone at 663 nm.

Allantoinase was determined by the diphenylformazan method (72), as in Chapter I. Glyoxylate production by allantoicase was measured by coupling to lactate dehydrogenase (73) or by the diphenylformazan method (72). The substrate was 10 mM allantoic acid in either 25 mM phosphate buffer (pH 7.4) or 20 mM Tricine (pH 7.8). Reactions were incubated 30 minutes at 30°C and stopped with the phenylhydrazine reagent for the diphenylformazan determination. Urea production was measured by ammonia formation coupled to urease (Sigma) added in excess to reactions carried out in Conway microdiffusion dishes (74). The reactions were stopped with saturated K_2CO_3 and ammonia determined (74) with Nessler's reagent (Sigma) after diffusion for 2 hours at 30°C.

Thin layer chromatography was done on glass cellulose plates containing fluorescent indicator in 7 ether:2 formic acid:1 water. Ureide groups were stained with p-dimethylaminobenzaldehyde (75).

For the radioactive assay for allantoicase, 0.8 ml 2-[^{14}C]uric acid in 0.1 M CHES (pH 9.0, Sigma) was incubated with 2 units uricase (Sigma) for 30 minutes at 25°C. The plant or enzyme sample was then added and the reaction was brought to a volume of 1.0 ml in a small vessel containing 0.4 ml 80% hyamine hydroxide in the center well. The reaction was incubated at 25°C, stopped with 0.2 ml 2 N HCl, and allowed to diffuse for 12 hours before counting the [^{14}C]CO_2 absorbed by the hyamine hydroxide.

RESULTS AND DISCUSSION

1. Isolation of Organelles

Isolation of intact peroxisomes from soybean leaves proved to be a difficult task. Good yields of peroxisomes have been previously obtained only from spinach and sunflower leaves, and only poor yields from other species (76). A comparison of catalase activity, the marker enzyme for peroxisomes, in soybean leaf extracts with the levels of activity previously found in spinach leaves (76) is presented in Table 6. Fewer total units of activity were measured in the soybean homogenate, and the specific activity was much lower than that in the spinach homogenate, indicating fewer units were extracted from soybean leaves than from an equivalent amount of spinach leaves. The percentage of the original catalase activity found in the 6000 g pellet, which was applied to the sucrose density gradient, was only 4% for soybean leaves, versus

TABLE 6

Comparison of Soybean and Spinach Leaf Extracts

For Catalase and Peroxisomal Isolation

	Catalase Activity	
Fraction	55 g Soybean leaves	50 g Spinach leaves
Homogenate, units	41,000	583,000
Total protein, mg	715	2,200
Specific activity	58	265
600 g pellet units	700	-
6000 g pellet units	1,600	175,000
% yield of previous step	4	30
total protein, mg	62	460
specific activity	26	380
Peroxisomal peak, units	800	96,800

Leaves were ground in a Waring blendor and the homogenate was filtered and centrifuged at 600 g. The supernatant was centrifuged at 6000 g. This pellet was resuspended and applied to a sucrose density gradient. Spinach data are taken from Tolbert et al. (1969) Plant Physiology 44:135-147 (76). Units are umol·min^{-1}.

30% for spinach leaves, as if a major part of the peroxisomes were broken during the centrifugation steps. The specific activity of catalase in the soybean leaf extract was less than one-tenth that of the spinach extract at this point. The yield of catalase activity in the peroxisomal fraction of the soybean leaf gradient was less than 1% of the yield on the spinach leaf gradient. Thus fewer peroxisomes were extracted from soybean leaves, and many more peroxisomes broke in the steps prior to the sucrose gradient. While adequate amounts of soybean leaf peroxisomes could be isolated for localization of the major enzyme components, those enzymes present at low levels might not be detectable.

Organelles from cowpea leaf extracts were also separated on sucrose density gradients. Even fewer intact peroxisomes were obtained from cowpea leaves than from soybean. Cowpea organelles were also separated on a zonal gradient, but the yield of peroxisomes was very low. Almost all of the catalase activity appeared in the soluble fraction, apparently from broken peroxisomes.

Different methods of grinding or chopping the leaves were tried, as well as the addition of 2.5% w/v Ficoll to the medium. None of these methods gave any improvement in the yield of intact peroxisomes. It was necessary to add at least 1 g insoluble polyvinylpolypyrrolidine per 10 g of leaves to prevent browning of the extracts due to oxidation of phenolic compounds in the leaves. Much better yields of peroxisomes were obtained from growth chamber-grown plants than from greenhouse plants.

2. Localization of soybean leaf allantoinase

Sorbitol density gradients were used in order to avoid the possible interference by sucrose in the diphenylformazan assay for allantoinase

(Chapter I). The organelle fractions were broader on the sorbitol gradients and much of the activity of the peroxisomal marker enzyme, catalase, overlapped the mitochondrial marker activity of cytochrome c oxidase, as seen in fraction 8, Figure 7. Intact peroxisomes of higher purity appeared at a density of 1.25 g/cc in fraction 12, in which the specific activity of catalase was 700 umol min^{-1} mg $protein^{-1}$. Traces of uricase were also found in the peroxisomal fraction, but the levels were very low, as expected in the leaves.

Allantoinase activity co-sedimented with cytochrome c reductase, the microsomal marker enzyme. Allantoinase specific activity in the microsomal fraction was 120 nmol min^{-1} mg $protein^{-1}$. Total allantoinase activity was 320 nmol min^{-1} gram fresh $weight^{-1}$, about half the level found in nodules per gram fresh weight (Chapter I). The specific activity of allantoinase in a total leaf extract was 30 nmol min^{-1} mg $protein^{-1}$. The K_m for allantoin was about 30 mM. The pH optimum of the enzyme reaction was 7.8 to 8.0. Allantoin rapidly hydrolyzed nonenzymatically above pH 8.5.

Soybean leaf organelles were also separated on sucrose density gradients (Figure 8). Part of the catalase activity was found in fraction 7, where the mitochondrial fraction was located, as marked by cytochrome c oxidase, but the highest specific activity of catalase, 900 umol min^{-1} mg $protein^{-1}$, was found in fraction 12, at a density of 1.25 g/cc. This density is typical for a peroxisomal fraction (1). Allantoinase again co-sedimented with the microsomal marker cytochrome c reductase at 1.18 g/cc. Allantoinase maximum specific activity was 70 nmol min^{-1} mg $protein^{-1}$. Sucrose gave only a slight reaction in the diphenylformazan assay for allantoinase when performed as specified

Figure 7. Separation of Soybean Leaf Organelles on a Sorbitol Density Gradient.

Organelles were first pelleted by differential centrifugation, resuspended, and applied to the sorbitol gradient. The gradient was fractionated from the top (fraction 1 has the lowest density).

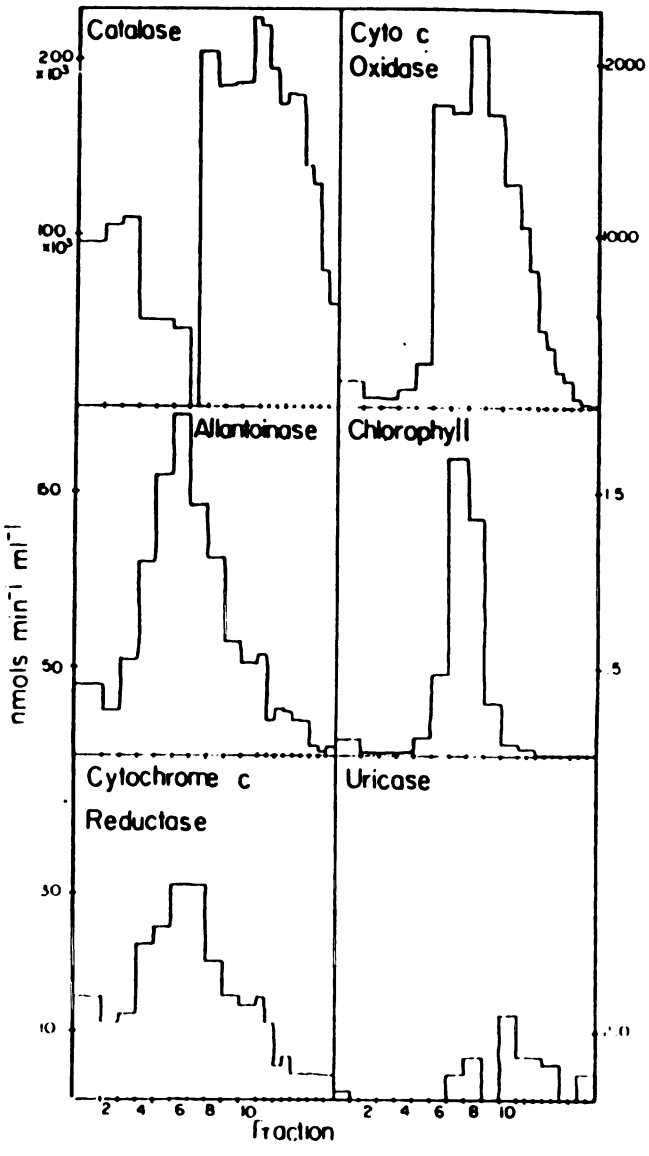


Figure 7. Separation of Soybean Leaf Organelles on a Sorbitol Density Gradient

Figure 8. Separation of Soybean Leaf Organelles on a Sucrose Density Gradient.

Organelles were first pelleted by differential centrifugation, resuspended, and applied to the sucrose gradient. The gradient was fractionated from the top (fraction 1 has the lowest density).

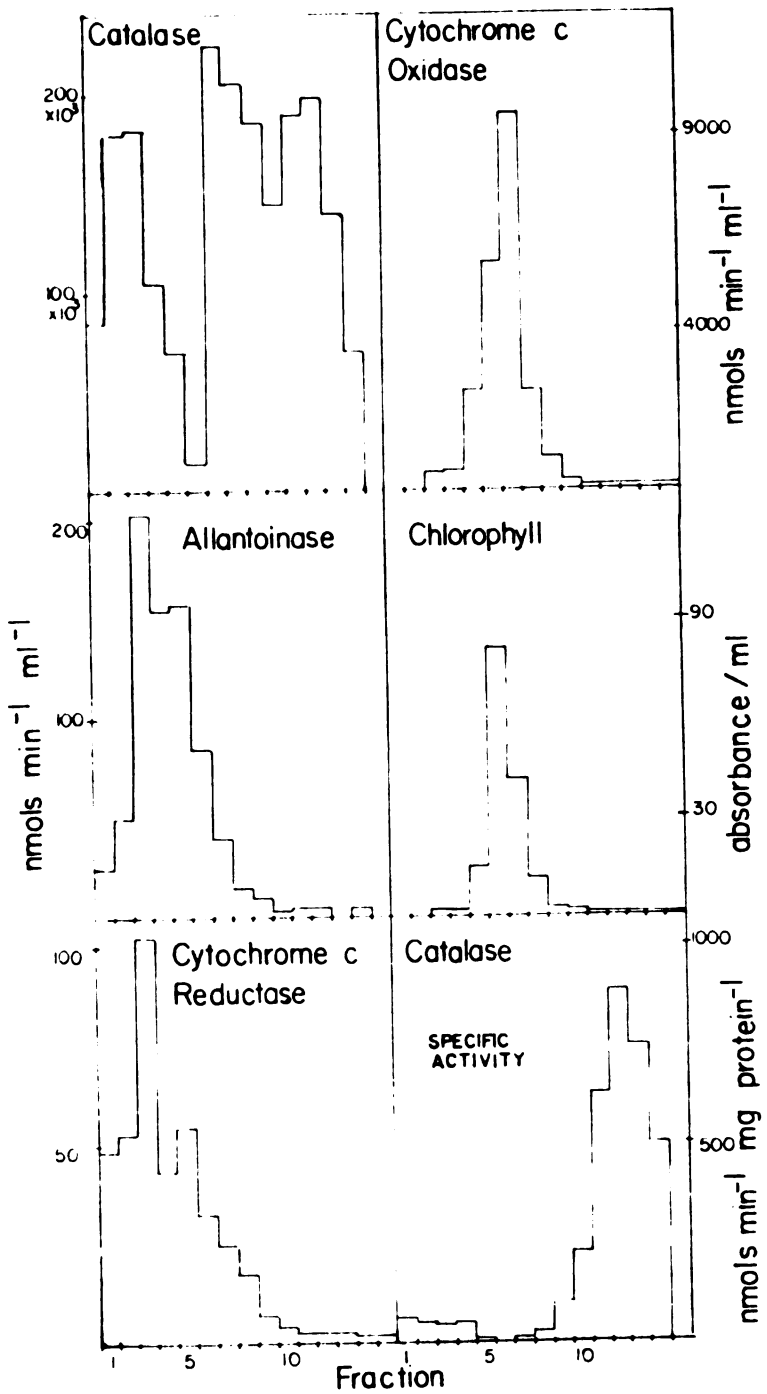


Figure 8. Separation of Soybean Leaf Organelles on a Sucrose Density Gradient

in the Methods section. The sucrose reaction was subtracted by running zero time controls.

Germinating soybean seedlings also degrade purines, presumably utilizing ureides stored in the cotyledon. While only traces of uricase were found in seedling peroxisomes, high levels of allantoinase occurred in the microsomal fraction (Figure 9).

In all of the soybean leaf or seedling gradients, allantoinase was found in the microsomal fraction, probably originating from the endoplasmic reticulum. These results contrast with previous reports of peroxisomal allantoinase (13, 14), but agree with localization of allantoinase in soybean nodules (Chapter I, 40).

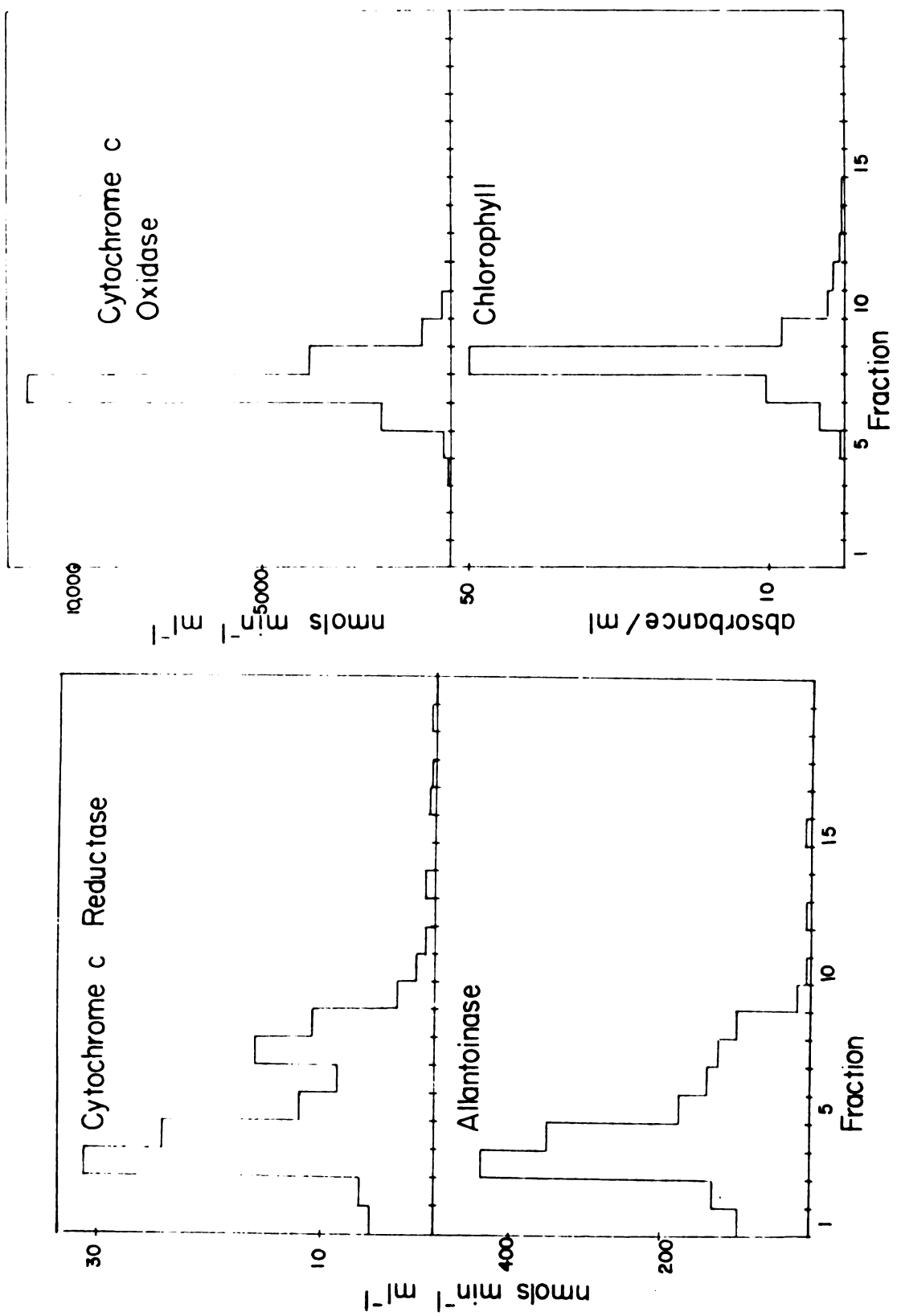
3. Soybean Allantoicase

Allantoicase activity may be determined by measuring either glyoxylate or urea, the products of the reaction. I chose first to look for glyoxylate, since enzymes involved in the metabolism of this compound are always found within peroxisomes (1). Several colorimetric or enzymatic methods for determination of glyoxylate are available.

Noguchi et al. (13) assayed allantoicase by the method of Gregory (73) and localized fish liver allantoicase in peroxisomes. In this assay (73), oxidation of NADH by lactate dehydrogenase is coupled to glyoxylate production by allantoicase (Chapter IV). However, allantoicase activity could not be detected in peroxisomal fractions or crude extracts of soybean leaves by this assay. Low endogenous rates were present that were not dependent on either allantoin or allantoic acid. Low levels of glyoxylate may be present in most leaf extracts and in the substrates, allantoin and allantoic acid, which decompose slowly to

Figure 9. Separation of Organelles From Soybean Seedling on a Sucrose Density Gradient.

Organelles were first pelleted by differential centrifugation, resuspended, and applied to the sucrose gradient. The gradient was fractionated from the top. Data in Figure 9a, b, and c are all from the same gradient.


Cytochrome c Reductase
Allantoinase
nmols min⁻¹ ml⁻¹
30
10
400
200
1
5
10
15
Fraction
Cytochrome c Oxidase
Chlorophyll
nmols min⁻¹ ml⁻¹
absorbance / ml
10000
5000
50
10
1
5
10
15
Fraction

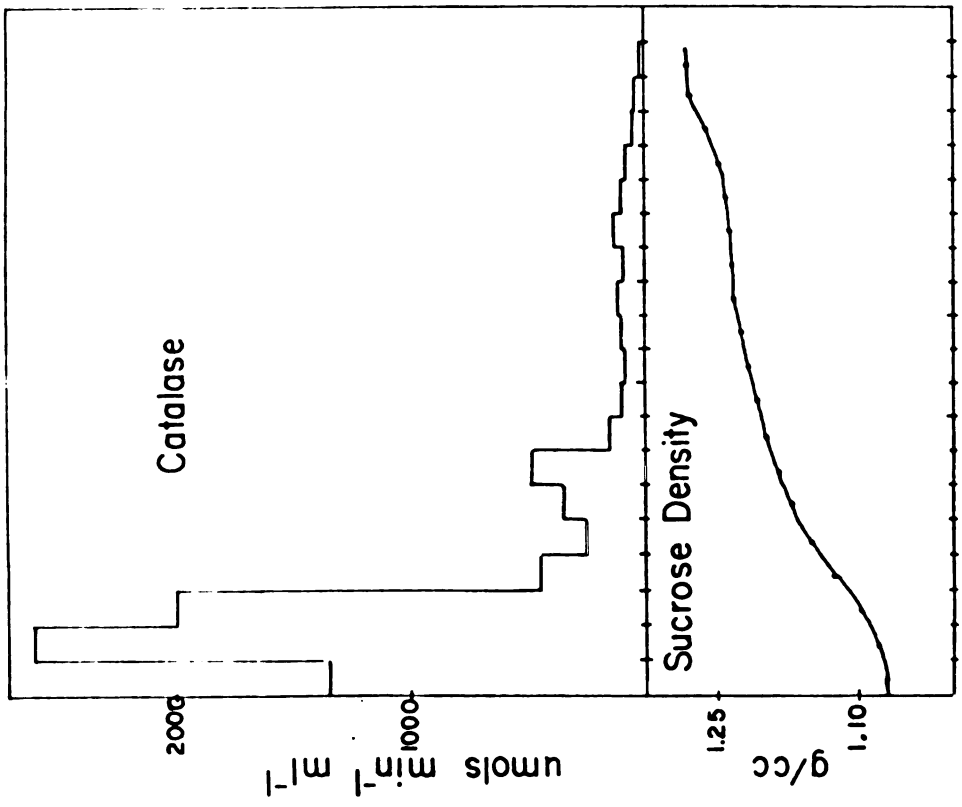

Catalase
Sucrose Density
2000
1000
umols min-1 ml-1
1.25
1.10
g/cc
1
5
10
15
Fraction

Figure 9b.

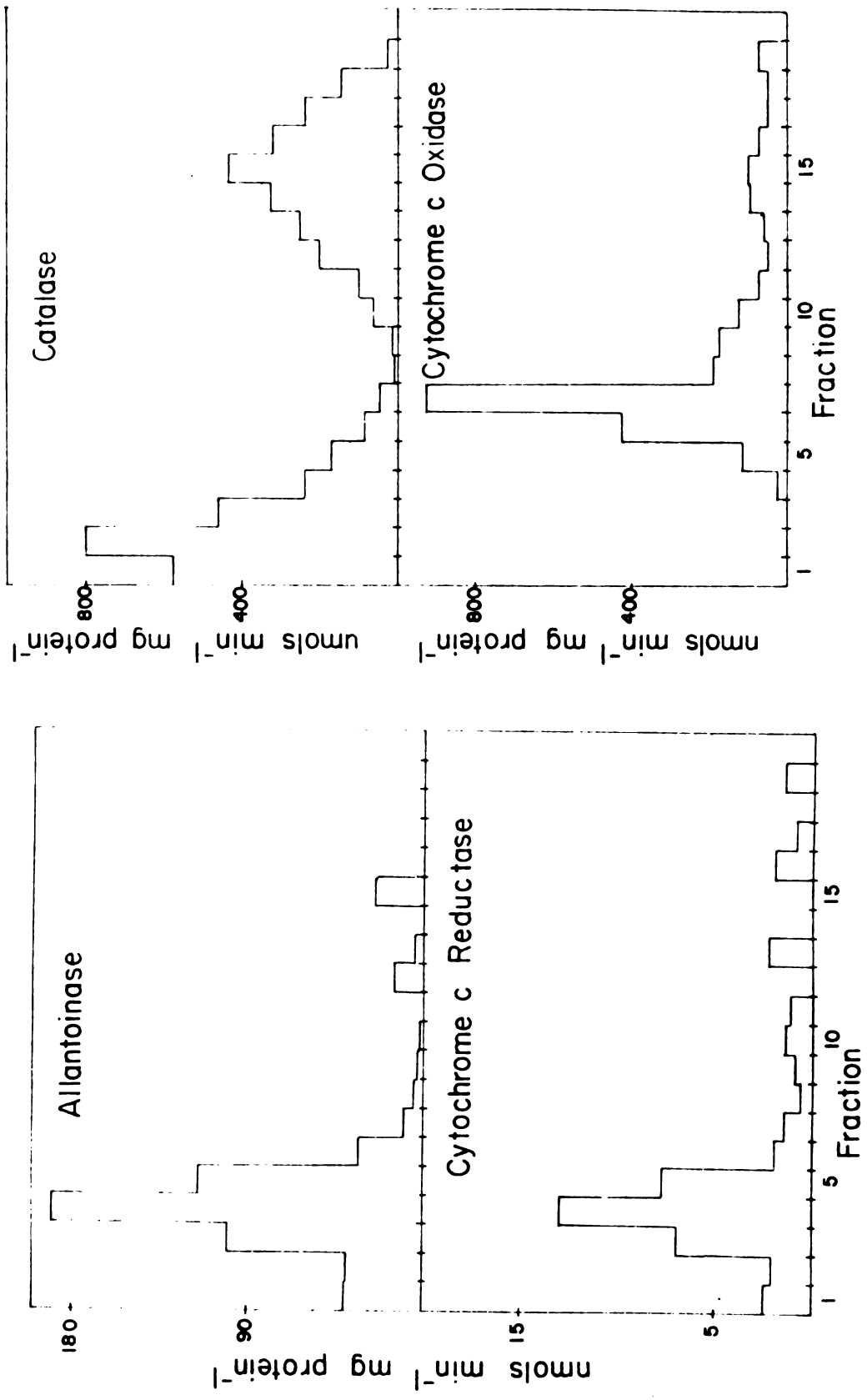

Allantoinase
Cytochrome c Reductase
Catalase
Cytochrome c Oxidase
nmols min⁻¹ mg protein⁻¹
μmols min⁻¹ mg protein⁻¹
Fraction
180
90
15
5
800
400
1
5
10
15

Figure 9c.

glyoxylate nonenzymatically. The addition of 0.1% Triton X-100 detergent and variations in pH from 7.0 to 8.5 did not reveal allantoicase activity in either cowpea or soybean leaves. The addition of 0.8% sodium deoxycholate or 0.2% digitonin did not effect allantoicase activity. The sensitivity of the coupled lactate dehydrogenase assay was tested by addition of glyoxylate. A pulse of 10 nmol of glyoxylate was the minimum amount required for a measurable activity. It is not known whether the expected allantoicase activity would be great enough to permit an accumulation of glyoxylate sufficient for detection by this assay.

Allantoicase activity might also be measured by coupling to the serine:glyoxylate aminotransferase already present in the peroxisomes. The products would be glycine and hydroxypyruvate, the latter of which could be coupled to hydroxypyruvate reductase monitored in turn by oxidation of NADH at 340 nm. This method is attractive because the two coupling enzymes should already be present in the peroxisomes. However, no allantoic acid-dependent activity was found in organelle fractions by this assay. The coupling enzymes were present in the peroxisomal fraction at a level sensitive enough to detect a pulse of 20 nmol of glyoxylate.

The most sensitive method for determining glyoxylate is the diphenyl formazan colorimetric method (72). When using this assay for allantoicase, I stopped the reactions with the phenylhydrazine reagent on ice, and proceeded with the color determination. High concentrations of chlorophyll, as in the chloroplasts, interfered slightly with the peak absorbance at 520 nm. Sucrose did not interfere with this assay for allantoicase, since the reactions did not have to be boiled. As

described earlier (Chapter I), sucrose is a problem in the allantoinase reaction which must be boiled in order to hydrolyze allantoic acid to glyoxylate.

No allantoicase activity could be detected in total leaf extracts or peroxisomal fractions with the diphenylformazan assay for glyoxylate. Both allantoin and allantoic acid were tried as substrates, as well as various detergents and buffers. A spectrophotometric assay for allantoicase was also attempted, measuring the phenylhydrazone of glyoxylate at 324 nm, but no allantoicase activity was detected. Low rates of activity (30 nmol min^{-1} ml^{-1}) were occasionally found in the chloroplast fraction, but this activity was not substrate dependent, and was probably due to an artifact caused by the high concentration of chlorophyll. Allantoicase activity did not occur in chloroplasts of seedlings, which were shown to hydrolyze allantoin in vivo (see Section 4). The above diphenylformazan assay for soybean allantoicase was performed under the same conditions, including the addition of magnesium chloride, which were successfully used to measure allantoicase from fish liver (Chapter IV). Since this endpoint assay is sensitive enough to detect low levels of activity, the lack of activity in soybean leaves may indicate that the pathway or the enzymes in soybean tissues are different from those in fish liver.

Allantoicase activity has been reported in extracts of leaves of nitrate grown bushbeans (36). The assay was unusual in that a small amount of phenylhydrazine was present during the reaction to trap glyoxylate produced. The diphenylformazan determination was performed after the reaction was stopped on ice. I found no allantoic acid-dependent activity in soybean leaf or seedling extracts with this assay, although artifactual rates in the absence of substrate were observed.

Another possible method of detecting allantoicase would be to measure the urea produced by the reaction. The urea production could be coupled to urease, and the ammonia determined by Nessler's reagent. It was necessary to microdiffuse the ammonia from the reaction chamber before carrying out the color reaction, which is sensitive to interference by many compounds (74). The reaction was stopped with strong base, driving off the ammonia, which was absorbed by acid in an inner compartment of the reaction chamber. Using this procedure, no allantoicase activity was detected in peroxisomal fractions or total extracts from soybean leaves, with uric acid, allantoin, or allantoic acid as substrates. In fact, the peroxisomal extracts incorporated part of the background ammonia present in the reagents. No urease activity was found in leaf extracts by this method.

Alternate pathways for allantoin metabolism exist in micro-organisms. Allantoin is fermented by Streptococcus allantoicus through a series of intermediates including allantoic acid, glyoxylurea, and carbamyloxamic acid, yielding oxamic acid, ammonia, ATP, and NADH as products (77). This pathway occurs only in the presence of NAD, $MgSO_4$, and phosphate or arsenate. In the absence of these cofactors, allantoic acid is degraded to glyoxylate and urea. Activity of the enzymes of this pathway could be determined by measurement of oxamate (78) or reduction of NAD at 340 nm. Although detectable levels of oxamate were present in soybean leaf extracts, no allantoic acid-dependent production of oxamate was found, nor was NAD reduced in the presence of allantoic acid and the above cofactors.

4. Radioactive Assay for Allantoicase

Ureides were separated by thin layer chromatography or high voltage electrophoresis and stained with p-dimethylaminobenzaldehyde (75), which gives a yellow color for allantoin, allantoic acid, or urea. Uric acid was detected by fluorescence. Soybean leaf extracts contained high levels of allantoic acid and urea when separated by either method. This accumulation may indicate that the allantoicase and urease reactions are limiting steps in the pathway, allowing a build-up in the pools of allantoic acid and urea.

A radioactive in vivo assay was employed for allantoinase and allantoicase. [2-^{14}C]Uric acid was converted to [2-^{14}C]allantoin using excess uricase purchased from Sigma. The conversion reaction, monitored at 293 nm for absorption by uric acid, was essentially complete, since the radioactive allantoin was free of uric acid when analyzed by thin layer chromatography. Any remaining uric acid would not have interfered with the assay, although the specific activity of the allantoin would have been lower than expected. Soybean pods, leaves, or seedlings were incubated with the [2-^{14}C]allantoin in closed vials containing a small center cup filled with 80% hyamine hydroxide. [^{14}C]CO_2 from completely hydrolyzed allantoin was trapped in the base. When extracts were assayed, the reaction was stopped with acid, driving off the [^{14}C]CO_2. This assay requires the action of all three enzymes in the pathway; allantoinase, allantoicase, and urease. Since the [2-^{14}C]uric acid contained a small amount of [^{14}C]urea, control reactions of substrate plus purchased uricase and urease were always run as well as nonenzymatic controls, and any background counts found were subtracted from the enzyme reactions.

Since several hours were necessary for uptake of substrate in the *in vivo* assay, and the uptake was not quantitative, it is not possible to determine enzyme rates from these data, and many factors such as differences in rate of uptake may have affected the results. However, the data does indicate that a pathway for degradation of allantoin does exist in these plants, although it has not been possible to demonstrate activity of the pathway *in vitro*.

With extracts of soybean pods only very low levels of allantoin degradation in the radioactive assay were observed. When samples of these reactions were separated by high voltage electrophoresis, the majority of the label was in urea. In view of the recent report (42) that urease is not present in the shoot, it may be concluded that the final step in the pathway did not occur in these reactions. However, the presence of label in urea indicates possible activity of allantoinase and allantoicase. Whole pods also showed very low activity for the whole pathway *in vivo* (Table 7).

Earlier work suggested allantoin was hydrolyzed to glyoxylate in wheat seedlings (38). Wheat seedlings should contain the allantoin pathway, since ureides are stored in many seeds (22), even though mature wheat plants do not contain detectable ureides. Seedlings were also very useful for these experiments because of their small size and high rate of uptake of substrate through the roots. Using the radioactive assay procedure, wheat seedlings degraded 4 to 9% of the labeled allantoin to $[^{14}C]CO_2$ (Table 7).

Soybean seedlings had much higher levels of allantoin hydrolysis *in vivo*, degrading 60% of the allantoin in either light or dark (Table 7). The seedlings probably did not take up all of the $[^{14}C]$ allantoin

surrounding the roots. An extract of soybean seedlings degraded only a small fraction of the [^{14}C]allantoin, in agreement with earlier failures to detect the final steps of this pathway in soybeans *in vitro* (Section 3).

All attempts to measure the production of either glyoxylate or urea as a result of allantoicase activity *in vitro* have met with little or no success. Results of *in vivo* experiments indicate that some pathway for degradation of allantoin does exist in soybean plants, but the identity of the intermediates and products, the level of activity of the latter enzymes in the pathway, and the intracellular location of the pathway remain to be determined. Allantoinase activity was present in all soybean tissues examined, including nodules, leaves, and seedlings, and was always associated with the endoplasmic reticulum.

Table 7

Allantoicase Activity in Soybean Seedlings

	[2-^{14}C]allantoin supplied	[^{14}C]CO released	Degradation
	cpm	cpm	%
Soybean seedlings			
24 hours light	174,000	98,600	57
4 hours light	380,000	209,000	55
4 hours dark	380,000	226,000	60
extract	380,000	10,000	2.6
Soybean pods	828,000	6,000	0.7
	225,000	7,850	3.5
Wheat seedlings	440,000	16,400	3.7
	225,000	20,000	8.9

Allantoicase was assayed *in vivo* by the radioactive method. Controls were run to determine the amount of [^{14}C]urea contaminating the [^{14}C]allantoin, as well as any nonenzymatic breakdown, and these low background levels were subtracted. *In vivo* reactions were incubated for 24 hours to allow uptake, degradation, and absorption of [^{14}C]CO_2, except where otherwise specified. A soybean seedling extract containing 2 mg protein was incubated for 2 hours and stopped with HCl. Specific acitivity of the [2-^{14}C]allantoin was 1000 cpm·nmol^{-1}.

CHAPTER IV

INTRACELLULAR LOCALIZATION OF PURINE DEGRADATION IN FISH LIVER

INTRODUCTION

Previous reports disagree as to the intracellular location of some of the enzymes of purine degradation in the lower vertebrates, including fish, amphibians, and birds. Xanthine oxidase was reported only in the soluble fraction from fish liver (13), although no data was shown, while xanthine dehydrogenase from chicken liver and kidney was peroxisomal (14), but could not be detected in frogs (14). Enzyme activities in this latter paper (14) were given as a percent of the total activity or as relative units to the mean activity of all the fractions, with no data on activity or specific activity. Xanthine oxidase and xanthine dehydrogenase appear to be two forms of the same protein (15). Uricase is always in the peroxisomal fraction from all tissues (13,14), and is the final enzyme of the pathway present in chickens (14).

Allantoinase was reported to be present in liver peroxisomes from frogs, but was in the soluble fraction in frog kidney (14). It was necessary to include $MgCl_2$ in the grinding media in order to keep allantoinase in the peroxisomal fraction of frog liver (14). In another report (13), uricase and allantoinase appeared to be located in the peroxisomal matrix and allantoicase in the peroxisomal membrane from fish liver. While uricase and allantoinase were present also in the soluble fraction as a result of peroxisomal breakage, allantoicase was located only in the peroxisomes (13). Allantoicase was reported to be soluble in frog liver and kidney, although no assay or data were given (14).

In view of the fact that my results with plant tissues located allantoinase in the endoplasmic reticulum (Chapter III), I have reexamined

previous work on this enzyme. The allantoinase assay of Noguchi et al. (13) was dependent on glyoxylate reduction by excess lactate dehydrogenase. When allantoin was the substrate, the activities of both allantoinase and allantoicase would be required to produce glyoxylate in this coupled assay. Soluble allantoinase should not have been detected, since allantoicase was present in the peroxisomal fraction only. The authors gave no explanation for the reported allantoinase activity shown in the soluble fraction and did not mention any other assay method. Obviously such discrepancies between the data and the assay cast doubt on the conclusions reached by these authors. Indeed, such a coupled assay, dependent on the presence of both enzymes, is not appropriate for a localization experiment for either enzyme. In my studies on allantoin metabolism, I have used a direct diphenylformazan assay for the separate catalytic products, allantoic acid and glyoxylate (72). Thus allantoinase and allantoicase activity can be measured independently.

Since questions remained regarding the location of purine degradation in animal cells, and allantoicase activity could not be detected in soybean leaf peroxisomes, I investigated purine degradation in fish liver. I hoped to clarify the localization of allantoinase, which was microsomal in all soybean tissues, and to learn more about optimum conditions for assaying allantoicase. Data and experience with the fish allantoicase would be useful in searching for this enzyme in soybean leaves, if indeed the same enzyme were present.

MATERIALS AND METHODS

Bluegill-green sunfish hybrids were the gift of Jay Gouch of the Fisheries and Wildlife Department. Rainbow trout were provided by Dr.

J. Hoffert from the Physiology Department. Madagascar mouth breeders were purchased from a local pet shop.

Grinding media, tissue, and gradient varied between experiments as detailed below. Minced liver was extracted once with a Tektron homogenizer, filtered through six layers of cheesecloth or Miracloth, and centrifuged at 300 g for five minutes. The supernatant was applied to the gradient described below, centrifuged for three hours at 106,900 g, and then fractionated from the top of the gradient.

Gradient 1: Six g liver from two bluegill-green sunfish hybrids was homogenized in 10% sucrose in 0.1 M Tricine, (pH 7.5) and applied to a step gradient of 3 ml of 2.3 M sucrose, 5 ml of 1.9 M sucrose, 7 ml of 1.8 M sucrose, 7 ml of 1.75 M sucrose, 7 ml of 1.7 M sucrose, 7 ml of 1.5 M sucrose, 6 ml of 1.3 M sucrose, 4 ml of 1.0 M sucrose, and 4 ml of 0.83 M sucrose; all prepared in 0.1 M Tricine (pH 7.8).

Gradient 2: 1.6 g liver (bluegill-green sunfish) was homogenized in 8.5% sucrose in 1 mM phosphate (pH 7.5) and applied to the above sucrose gradient, prepared in 1 mM phosphate (pH 7.5).

Gradient 3: 1.6 g liver from the same fish as gradient 2 was homogenized in 15% sorbitol, 0.1 M Tricine (pH 7.8), 10 mM KCl, 1 mM $MgCl_2$, and 10 mM EDTA (Beever media, 79) and applied to a gradient identical to gradient 2.

Gradient 4: 1.1 g liver from two Madagascar mouth breeders was homogenized in 8.5% sucrose in 1 mM phosphate (pH 7.5) and applied to a gradient identical to gradient 2.

Gradient 5 and 6: 4.4 g liver from two rainbow trout was homogenized in 10 ml Beever media and divided between two gradients as above. Gradient 5 contained 0.1 M tricine (pH 7.8); gradient 6 contained 1 mM phosphate (pH 7.5).

Gradients 7 and 8: 2.9 g of rainbow trout liver was homogenized in 0.25 M sucrose, 20 mM Tricine (pH 7.8), 20 mM KCl, 1 mM $MgCl_2$, and 20 mM EDTA (medium 7) and divided between two gradients. Gradient 7 was a 48 ml linear gradient of 0.75 M to 2.0 M sucrose on top of a 3 ml pad of 2.3 M sucrose, all prepared in 20 mM Tricine (pH 7.5). Gradient 8 was identical, except for the addition of 1 mM $MgCl_2$.

Gradients 9, 10 and 11: 3.0 g of rainbow trout liver was homogenized in 10 ml medium 7 and divided between three linear gradients of 1.0 M sucrose to 2.1 M sucrose, over a 3 ml 2.3 M sucrose pad. Gradient 9 was prepared in 20 mM Tricine (pH 7.5). Gradient 10 was prepared in 5 mM Tricine (pH 7.5) plus 1 mM $MgCl_2$. Gradient 11 was prepared in 20 mM Tricine (pH 7.5) plus 2 mM dithiothreitol.

Catalase, uricase, NADH-cytochrome c reductase, cytochrome c oxidase, xanthine dehydrogenase, and allantoinase were assayed as described in Chapter I (40).

Allantoicase activity was measured in the presence of 10 mM allantoic acid in 25 mM phosphate (pH 7.0) or 20 mM Tricine, (pH 7.5) at 30°C for 10 to 30 minutes. The reaction was stopped by the addition of the phenylhydrazine reagent (0.8 ml) for the diphenylformazan determination of glyoxylate (72). This determination differs from the allantoinase assay, in which the reactions must be boiled to hydrolyze the product allantoic acid to glyoxylate, which is then measured in the diphenylformazan determination.

RESULTS AND DISCUSSION

Organelles from the liver of three species of fishes were separated on sucrose density gradients by essentially the same methods used in

Table 8

Total Activity of Enzymes Involved in Purine Degredation in Fish Liver

	Soluble		Peroxisomal Fraction					
	Allantoinase		Uricase		Catalase		Allantoicase	
gradient	units	S.A.	units	S.A.	units	S.A.	units	S.A.
1*	150	-	60	-	700	-	15	-
2	510	92	30	140	220	1120	5	28
3	830	240	31	100	250	920	5	20
4*	400	106	12	45	770	6380	5	2.5
5*	2300	240	250	43	9470	1610	162	28
6	2100	230	74	74	2650	4260	9	7
7	1620	260	29	100	1870	7190	40	80
8*	2090	250	150	32	5470	1200	415	91
9	1150	-	21	-	1160	-	25	-
10	1400	212	34	34	1490	1490	33	33

Peroxisomes were purified on sucrose density gradients as specified in Methods. Approximately 1.5 to 2.0 g liver was used per gradient. Enzyme units are nmol min^{-1}, except catalase which is in umol min^{-1}, and S.A. are units·mg $protein^{-1}$. Allantoinase data is from the soluble fraction; no activity was present in the peroxisomal fraction. Uricase, catalase, and allantoicase data are from the peroxisomal fraction, except where an asterick (*) indicates the organelles aggregated into a single band.

isolation of plant organelles. In several cases, especially in the presence of high concentrations of buffer (0.1 M Tricine) or 1 mM $MgCl_2$, all of the organelles aggregated into a single fraction (Table 8, gradients 1, 4, 5), or the mitochondria and peroxisomes failed to separate (gradient 8). Better separation was obtained on gradients containing only 1 mM phosphate buffer, and no $MgCl_2$ (gradients 2, 3, 6). However, more allantoicase activity was found when higher concentrations of buffer or $MgCl_2$ were used (gradients 1, 5, 8). In all the gradients, including those in which organelles aggregated, allantoinase activity was only in the soluble fraction (Table 8, Figure 10).

Enzyme units and specific activities in the soluble or organelle fractions of several of the enzymes involved in purine degradation are given in Table 8. The enzyme activities given are from the peroxisomal fraction, except for allantoinase. Uricase, catalase, and allantoicase activities were highest in the peroxisomal fraction, while allantoinase was found only in the soluble fraction (Figure 10). Levels of uricase and allantoicase varied widely betwen experiments, and were highest in those cases in which the organelles aggregated (gradients 1, 5, 8). Levels of uricase and allaantoicase appeared to fluctuate in parallel, while the level of allantoinase was independent of the other purine oxidizing enzymes.

Allantoicase was difficult to localize since the conditions yielding the highest enzyme activity and stability caused all the organelles to aggregate into a single fraction. While allantoicase activity was very stable at high buffer concentration (0.1 M Tricine, gradient 1, 5) or in the presence of $MgCl_2$ (gradient 8, 10), very little activity was found on 1 mM phosphate gradients (gradients 2, 3, 4, 6). Although the

Figure 10. Isolation of Fish Liver Peroxisomes on a Sucrose Density Gradient.

The 300 g supernatent from a liver homogenate was applied to a sucrose gradient prepared in 20 mM tricine. The gradient was fractionated from the top. Figure 10a to 10d data are all from the same gradient.

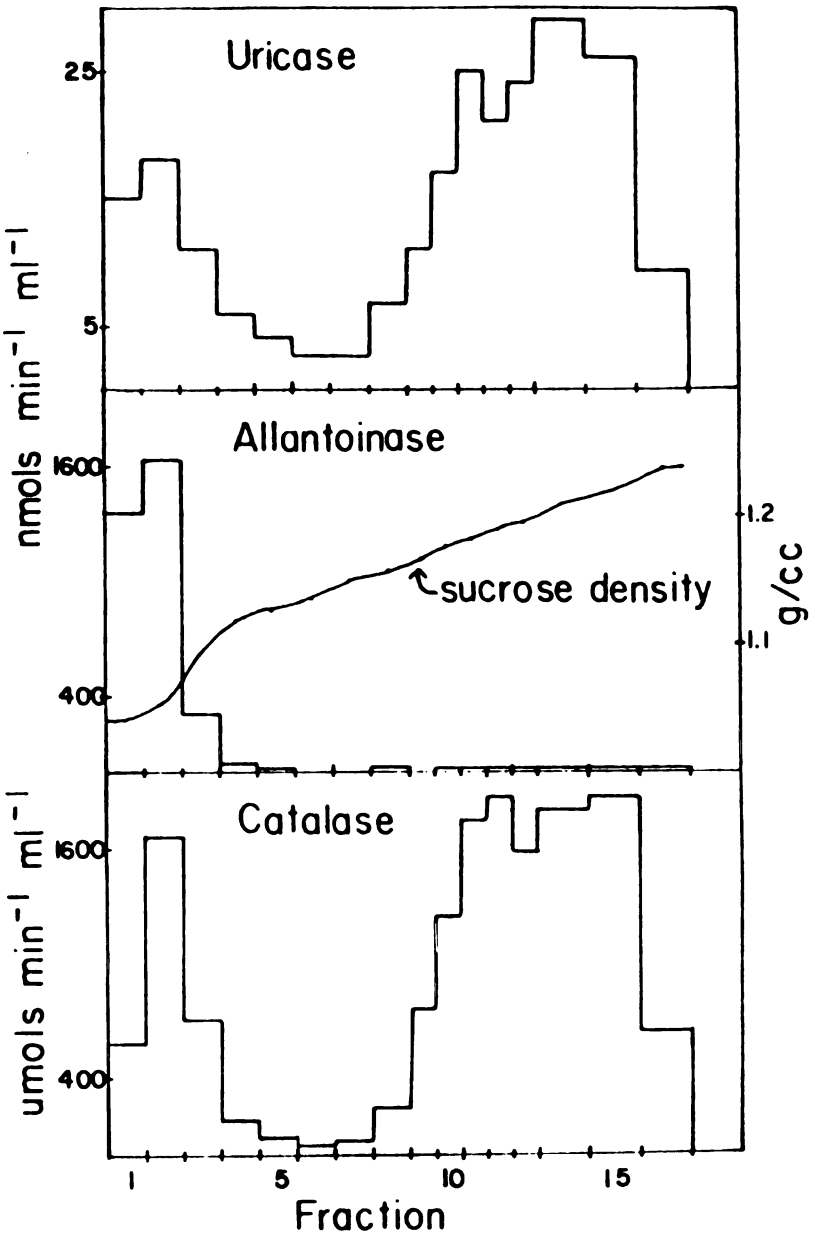


Figure 10. Isolation of Fish Liver Peroxisomes on a Sucrose Density Gradient.

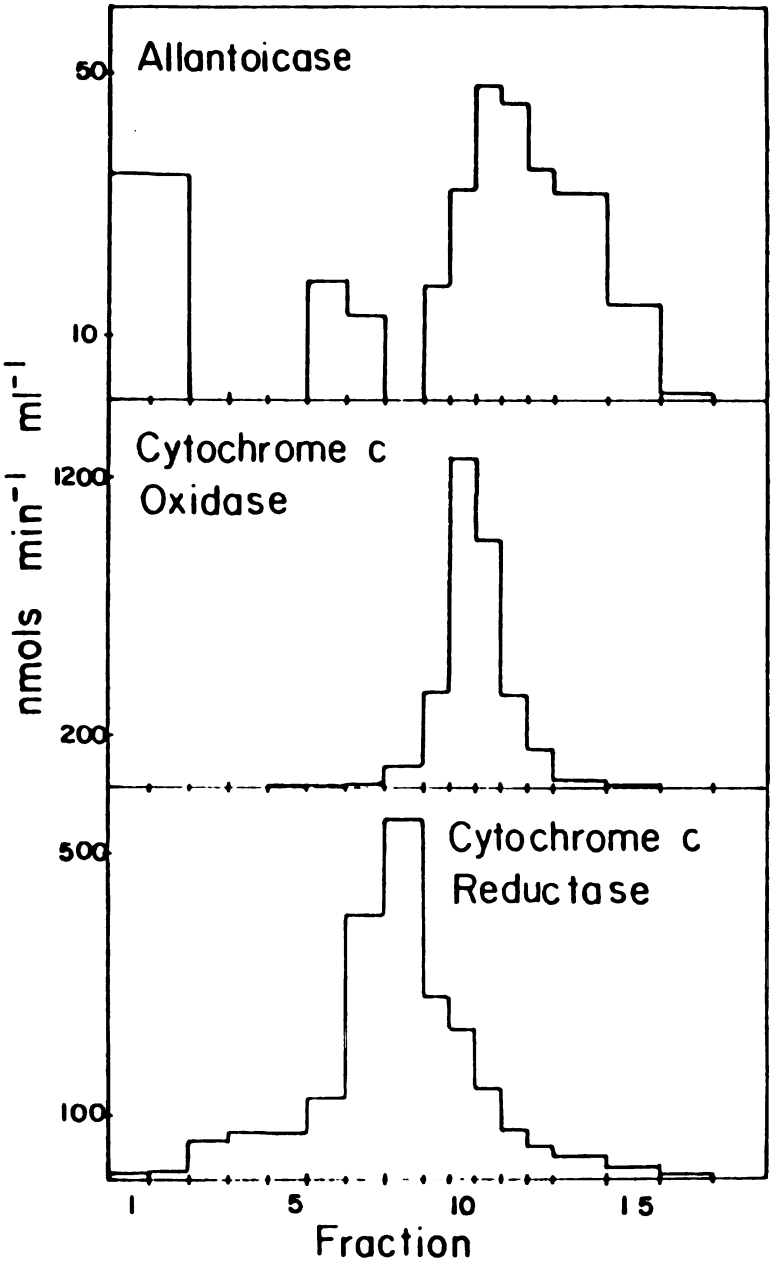

Allantoicase
50
10
Cytochrome c
Oxidase
1200
200
Cytochrome c
Reductase
500
100
nmols min⁻¹ ml⁻¹
1
5
10
15
Fraction

Figure 10b.

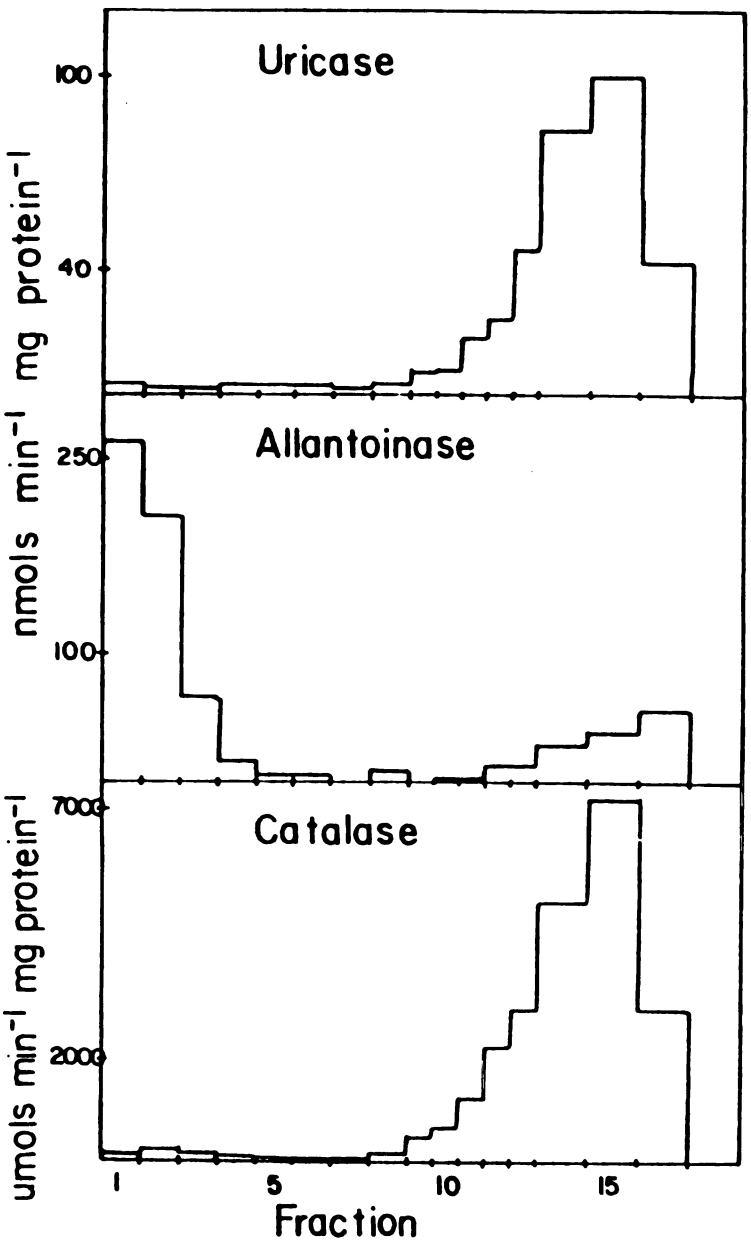

Uricase
100
40
nmols min⁻¹ mg protein⁻¹
Allantoinase
250
100
Catalase
7000
2000
umols min⁻¹ mg protein⁻¹
1
5
10
15
Fraction

Figure 10c.

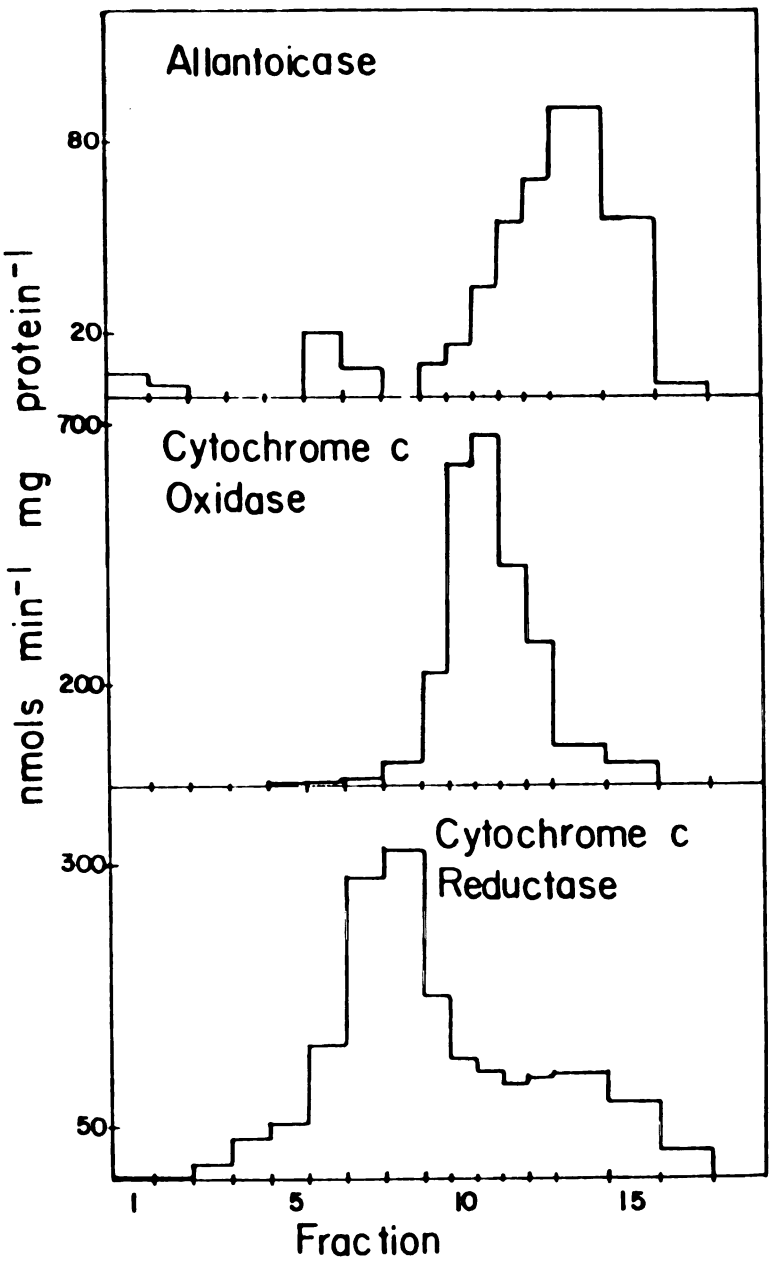

Allantoicase
80
20
Cytochrome c
Oxidase
700
200
Cytochrome c
Reductase
300
50
nmols min⁻¹ mg protein⁻¹
1
5
10
15
Fraction

Figure 10d.

same homogenate was applied to gradients 5 and 6, only gradient 5 (0.1 M Tricine) contained a high level of allantoicase in the organelle fraction, even though much better separation of organelles was achieved on gradient 6 with 1 mM phosphate. Gradients 7 and 8 are identical, except for the addition of 1 mM $MgCl_2$ to gradient 8. While both gradients contained equal amounts of allantoicase in the soluble fraction, gradient 8 contained 10 times as much activity on the gradient, in which the organelles unfortunately aggregated. Data from gradient 7 is presented in Figure 10. Gradients 9 and 10 received equal portions of the same homogenate, but differ in their composition, gradient 10 containing less buffer (5 mM Tricine) with the addition of 1 mM $MgCl_2$. In this case, both gradients contained about the same level of allantoicase activity and reasonable separation was obtained on gradient 10 ($MgCl_2$). Allantoicase again peaked in the peroxisomal fraction.

All the allantoicase data presented were obtained by the direct diphenylformazan assay for glyoxylate (72). All of the peroxisomal fractions were also assayed by the coupled lactate dehydrogenase assay of Noguchi *et al.* (13), but little or no activity was found. This assay is not as sensitive as the diphenylformazan assay, since the K_m of lactate dehydrogenase for glyoxylate is quite high (80). Maximum allantoicase activity was found between 10 to 20 mM allantoic acid with the diphenylformazan assay.

Since high allantoicase activity had been found when organelles aggregated into a single fraction, samples of all the organelle fractions from gradients 2 and 3 were pooled and assayed to see if some kind of interaction or synergism between different organelles were necessary for allantoicase activity. However, no activity was found in either mixture of organelles.

Allantoinase activity appeared only in the soluble fraction from all ten gradients, and was not present in the microsomal fraction, indicated by the marker enzyme NADH-cytochrome c reductase. This was surprising, since this enzyme was in the microsomal fraction from all soybean tissues, and had been reported in the peroxisomal fraction from fish liver (13). The hypothesis was considered that allantoinase might be contained in very light microsomes which did not sediment into the gradients. To test this, 1.0 ml of the soluble fraction of gradients 2 and 3 was diluted with 2 ml 25 mM phosphate buffer, pH 7.5, and centrifuged at 145,000 g for 1 hour. The supernatent of both samples appeared biphasic, with an upper cloudy layer and a clear lower layer. These were removed separately, and the pellet was resuspended in 1.0 ml buffer. These three fractions from each tube, as well as the original soluble fraction from each gradient were assayed for allantoinase activity (Table 9). Almost all of the allantoinase activity remained in the supernatent fractions, consistent with the probability that this enzyme was not contained in microsomes. The possibility remains that allantoinase is only loosely bound to fish microsomes and was released under the homogenization conditions for these experiments. It is not considered likely that the soluble allantoinase came from broken peroxisomes, since no allantoinase activity was found in intact peroxisomes, which were recovered in good yield on several of the gradients (Figure 10).

Gradients 5 and 6 were used for characterization of allantoicase. Phsophate buffer (25 mM) was not inhibitory in the assay, and gave values equal to those obtained using 25 mM Tricine. In several instances samples lost all allantoicase activty when diluted 1:10 with 1 mM

Table 9

Tests for the Localization of Allantoinase in the Soluble Fraction by Centrifugation

gradient 2	Allantoinase units
	$nmol \cdot min^{-1}$
soluble fraction from gradient	309
upper supernatent	130
lower supernatent	168
pellet	6.6
% recovery in supernatents	98%
gradient 3	
soluble fraction from gradient	696
upper supernatent	242
lower supernatent	334
pellet	5.0
% recovery in supernatents	83%

One ml of the soluble fraction from the gradient was diluted with 2 ml of 25 mM phosphate buffer (pH 7.5) and centrifuged at 145,000 g for 1 hour. The supernatent was divided about equally into upper and lower fractions, and the pellet was resuspencded in 1 ml buffer.

phosphate buffer and incubated for one hour. Higher rates of activity were found using smaller samples of enzyme, as shown in Table 10. It is not likely that lower rates with increasing enzyme samples were caused by substrate depletion, since 10 mM allantoic acid or 10 umol of substrate was present in the assay, and fewer than 100 nmol of product were produced. The nonlinearity of activity versus enzyme concentration may have been caused by product inhibition or limiting amounts of some cofactor.

Addition of $MgCl_2$ to the reaction caused a dramatic increase in allantoicase activity (Table 11). The gradient fractions containing the highest allantoicase activity were chosen for this experiment, since the soluble fraction contained $MgCl_2$ from the homogenization media. As mentioned above, greater initial activity was present in the aggregated organelle fraction from gradient 5, which contained 0.1 M tricine, than in the peroxisomal fraction of gradient 6, which contained only 1 mM phosphate. In fact, the allantoicase activity initially present in the peroxisomal fraction of gradient 6 (9 nmol min^{-1} ml^{-1}) was almost completely gone (0.4 nmol min^{-1} ml^{-1}) when this experiment was performed, but activity was restored by the addition of $MgCl_2$. Maximum stimulation by $MgCl_2$ occured at a concentration of 5 mM or greater, which was included in all further experiments with this enzyme. These results provide a possible explanation for the instability of this enzyme in dilute buffer, and low activity on 1 mM phosphate gradients.

The pH optimum for allantoicase was approximately 7.0 (Table 12). This experiment was complicated by the instability of allantoic acid at lower pH values. The nmol of product formed by substrate incubated

Table 10

Non-linearity of Allantoicase Assay

Enzyme Aliquot	Activity
	nmol min^{-1} ml^{-1}
10 ul	102
20 ul	54
30 ul	51
40 ul	33

Aliquots of the soluble fraction of gradient 5 were assayed for 5 minutes by the diphenylformazan assay (72). The enzyme samples contained 1 mM $MgCl_2$ from the grinding medium, but $MgCl_2$ was not added to the assay.

Table 11

Stimulation of Allantoicase by Magnesium Chloride

	Allantoicase activity	
$MgCl_2$	gradient 5	gradient 6
	$nmol \cdot min^{-1} \cdot ml^{-1}$	
none	114	0.4
0.1 mM	142	2.6
1.0 mM	190	2.6
5.0 mM	311	10.2
10.0 mM	270	9.7
10.0 mM	289	10.6

10 ul of the organelle fraction from gradient 5 or 20 ul of the peroxisomal fraction of gradient 6, along with the indicated addition of $MgCl_2$ to the substrate, were assayed for allantoicase activity.

Table 12

Effect of Buffer and pH on Allantoicase Activity

Buffer	pH	Nonenzymatic Reaction	Total Reaction	Enzyme rate
		nmol product·20 min^{-1}		nmol min^{-1} ml^{-1}
Acetate	4.7	17.2	12.4	-16
MES	6.2	4.3	16.0	39
Phosphate	6.8	4.2	19.5	51
MOPS	7.2	0.8	17.6	47
TES	7.5	2.5	15.8	45
Tricine	8.2	3.6	13.3	32
CHES	9.3	1.8	5.5	12

Allantoic acid (15 mM) was prepared in the various buffers with 20 mM $MgCl_2$, and incubated both with and without 15 ul enzyme for 20 minutes at 30°C. The reaction was terminated by the addition of the phenylhydrazine reagent and nmol glyoxylate determined by the diphenylformazan reaction. The net enzyme rate was calculated by the difference between (nmol from the enzyme reaction minus nmol from the nonenzymatic reaction) divided by (20 minutes X 15 ul enzyme). Control reactions indicated the various buffers had little intrinsic effect on the reaction rate.

alone or with the addition of enzyme is shown in Table 12, along with the net enzyme rate calculated by subtraction of the non-enzymatic reaction.

The addition of pyridoxal phosphate had no effect on the allantoicase reaction. Dithiothreitol had a slightly inhibitory effect on the enzyme reaction.

Xanthine dehydrogenase or oxidase were not detectable in any of the fish liver extracts or gradients. This enzyme is known to be unstable, but sometimes may be stabilized by dithiothreitol (81). However, no activity was found in any of the fractions from a gradient containing 2 mM dithiothreitol, or in a sample of the homogenate to which dithiothreitol was added. These assays were performed immediately after fractionating the gradient, but the activity was apparently rapidly lost or there was very low activity in the species of fish used for these experiments. The assays were done at both 293 nm and 340 nm for xanthine oxidase and dehydrogenase, respectively, and were quite sensitive when checked with purchased xanthine oxidase. Since previous workers (13) provide no actual data for this enzyme, little can be concluded at this point regarding the localization of xanthine dehydrogenase. Xanthine dehydrogenase was in the soluble fraction in soybean nodules (40).

SUMMARY

Purine degradation serves very different functions in plants and animals. Animals degrade purines to a form which is convenient for excretion, since animals have no further use for these compounds. In the plant world, purine degradation products are used by many legumes as an efficient form in which to transport nitrogen from the nodule for utilization in other portions of the plant. As different as these purposes are, many similarities exist between the pathways of purine degradation in plants and animals. The most important of these is the compartmentation of at least part of the pathway in peroxisomes. The uricase reaction always takes place within peroxisomes, where catalase is also present to destroy the hydrogen peroxide produced by uricase. Allantoicase has been localized in peroxisomes of animals such as fish, which excrete urea. This enzyme is not present in nodule peroxisomes, since the pathway ends with the allantoinase reaction in soybean nodules. Nodule peroxisomes appear to function only in purine degradation, since they do not contain many of the enzymes normally found in glyoxysomes or leaf peroxisomes. The nature of the final reactions of purine degradation in soybean leaves has not been elucidated, but an enzyme such as allantoicase may be involved, and might be expected to be compartmented within leaf peroxisomes if glyoxylate is a product of the reaction.

The allantoinase reaction is unusual in that it is localized in different compartments in plants and animals. Allantoinase is associated with the endoplasmic reticulum in soybean nodules, leaves and seedlings. The enzyme in fish liver is found in the soluble fraction. This

result remains an enigma, since the enzymes preceding and following allantoinase, uricase and allantoicase, are localized in peroxisomes, and it is not clear why allantoinase should not also be localized in peroxisomes. It is not considered likely that allantoinase was released from the peroxisomal fraction during these experiments, since none of the peroxisomal enzymes, uricase, catalase, and allantoicase followed a distribution similar to that of allantoinase. The association of the plant allantoinase with the endoplasmic reticulum may play a role in the excretion of allantoic acid from the nodule cells in which it is produced.

Compartmentation in the soybean nodule is further complicated by separation of nitrogen metabolism between two cell types, the uninfected cells and those infected with bacteria. It is well known that nitrogen is symbiotically fixed into ammonia in the infected cells. The peroxisomal reactions uricase and catalase, as well as allantoinase from the endoplasmic reticulum, were found only in the uninfected cell fraction. It may be concluded that ureide production is the exclusive function of the uninfected cells, and these cells are also the exclusive location of nodule peroxisomes. The cellular location of the reactions preceding uricase has not been resolved. Purine synthesis has been localized in the plastid (Appendix). Some of the enzymes associated with plastids, as well as several others whose products are required for purine synthesis, were present at a greater specific activity in the uninfected cell fraction. However, further work is necessary to establish the hypothesis that purine synthesis leading to ureide formation takes place in the uninfected cell fraction, and to determine what metabolite(s) is transported between infected and uninfected nodule cells.

APPENDIX

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Subcellular organization of ureide biogenesis from glycolytic intermediates and ammonium in nitrogen-fixing soybean nodules

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Abstract. Subcellular organelle fractionation of nitrogen-fixing nodules of soybean (*Glycine max* (L.) Merr.) indicates that a number of enzymes involved in the assimilation of ammonia into amino acids and purines are located in the proplastids. These include asparagine synthetase (EC 6.3.1.1), phosphoribosyl amidotransferase (EC 2.4.2.14), phosphoglycerate dehydrogenase (EC 1.1.1.95), serine hydroxymethylase (EC 2.1.2.1), and methylene-tetrahydrofolate dehydrogenase (EC 1.5.1.5). Of the two isoenzymes of aspartate aminotransferase (EC 2.6.1.1) in the nodule, only one was located in the proplastid fraction. Both glutamate synthase (EC 1.4.1.14) and triosephosphate isomerase (EC 5.3.1.1) were associated at least in part with the proplastids. Glutamine synthetase (EC 6.3.1.2) and xanthine dehydrogenase (EC 1.2.1.37) were found in significant quantities only in the soluble fraction. Phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) was found mostly in the soluble fraction, although small amounts of it were detected in other organelle fractions. These results together with recent organelle fractionation and electron microscopic studies form the basis for a model of the subcellular distribution of ammonium assimilation, amide synthesis and ureide biogenesis in the nodule.

Key words. Ammonium assimilation – *Glycine* – Nitrogen fixation – Proplastid – Purine synthesis – Root nodule – Ureide

Introduction

It is now well established that a number of legume species, including soybean, assimilate most of the ammonium produced by nitrogen fixation into the "ureides", allantoin and allantoic acid (Herridge et al. 1978; McClure and Israel 1979). The ureides are produced in a pathway involving de novo purine biosynthesis followed by oxidation of the purine ring (Atkins et al. 1980; Schubert 1981; Boland and Schubert 1982). The subcellular location in peroxisomes, endoplasmic reticulum, and cytoplasm of the enzymes of purine oxidation has recently been presented (Hanks et al. 1981). Studies on the enzymology of purine biosynthesis and its ancillary processes in soybean nodules have demonstrated the presence of ribosephosphate pyrophosphokinase (Phosphoribosylpyrophosphate synthetase, PRPP synthetase; EC 2.7.6.1) and phosphoribosyl amidotransferase (EC 2.4.2.14), which catalyze two of the initial steps of purine biogenesis (Schubert 1981; Reynolds et al. 1982a) as well as phosphoglycerate dehydrogenase (EC 1.1.1.95), serine hydroxymethylase (EC 2.1.2.1) and methylene FH_4 (tetrahydrofolic acid) dehydrogenase (EC 1.5.1.5), which function in the production of glycine and N^5,N^{10}-methenyl FH_4, precursors for purine synthesis. The levels of these enzymes are much greater in soybean nodules than in nodules of lupin, a legume in which the main products of ammonium assimilation are amino acid amides rather than ureides (Reynolds et al. 1982b). All these enzymes have been shown in recent experiments to increase dramatically in specific activity in soybean nodules during the onset of nitrogen fixation and ureide production (Schubert 1981; Reynolds et al. 1982a).

The intracellular location of these enzymes has been investigated in view of the complexity and unusual structural nature of the nodule cell, and the compartmentation in different organelles of the purine-oxidizing enzymes (Hanks et al. 1981). Our experimental approach has been to fractionate nodule organelles on sucrose density gradients and measure levels of the key enzymes in all organelle fractions.

Abbreviations: FH_4 = tetrahydrofolic acid; PRPP = 5-phospho-α-D-ribose 1-pyrophosphate; PRPP synthetase = ribosephosphate pyrophosphokinase (phosphoribosylpyrophosphate synthetase)

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including all the enzymes heretofore mentioned and also those involved in assimilation of ammonium into amino acids and amides.

Materials and methods

[U-^{14}C]serine (2.15 GBq/mmol), [4-^{14}C]aspartate (1.89 GBq/mmol) and [U-^{14}C]glutamine (1.51 GBq/mmol) were obtained from the Radiochemical Centre, Amersham, U.K. Phosphohydroxypyruvate was generated from the dimethylketal derivative, which was obtained from Sigma Chemical Co., St. Louis, Mo., USA. Tetrahydrofolate, 5-phospho-α-D-ribose 1-pyrophosphate (PRPP), malate dehydrogenase (porcine heart cytoplasm), α-glycerophosphate dehydrogenase (type I) and 1 ml vials of NADH, NAD$^+$ and NADP$^+$ were obtained from Sigma. All other reagents were of analytical purity.

Three separate experiments were carried out, in which the conditions were varied according to the availability of soybean plants and attempts to stabilize different enzymes. Soybean plants (*Glycine max* (L.) Merr. cv. Amsoy 71) were grown from seeds (from Michigan Foundation Seed Corp., East Lansing, Mich., USA) inoculated with *Rhizobium japonicum* strain 3I1b 110 (U.S. Department of Agriculture, Beltsville, Md., USA) and grown in a growth chamber (under cool-white fluorescent and tungsten lamps at an energy fluence rate of 120 W m^{-2} with a 12-h photoperiod and a temperature of 35° C/day and 20° C/night; experiments 1 and 2) or greenhouse (during the months of August and September with additional light supplied for a 16-h photoperiod from paired Gro-lux and cool-white fluorescent lamps at fluence of 50 W m^{-2}, and a temperature of 35° C/day and 25° C/night; experiment 3) in Perlite (heat-treated volcanic rock; Therm-o-Rock, New Eagle, Pa., USA) and watered daily with nitrogen-free nutrient solution (Hubbeck et al. 1973). Three-week-old plants were used. Extracts from 3.3 g of nodules were fractionated on sucrose density gradients as described by Hanks et al. (1981). The grinding media contained 0.4 M sucrose, 0.1 M N-tris(hydroxymethyl)methyl glycine (Tricine) at pH 7.8, 10 mM KCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 1 mM $MgCl_2$, 25 μg ml^{-1} pyridoxal phosphate and 10 mg ml^{-1} fatty-acid-free bovine serum albumin. Dithiothreitol was also included in all grinding media at a concentration of 2.5 mM for experiment 1, and 20 mM for subsequent experiments. Reduced glutathione (5 mM) was added to the medium for experiment 2, to protect phosphoribosyl amidotransferase. Homogenates were layered on a 48-ml linear gradient of 0.75–2.0 M sucrose in 20 mM Tricine, pH 7.8, and 25 μg ml^{-1} pyridoxal phosphate, over a 3-ml bottom pad of 2.3 M sucrose. Dithiothreitol was also included in all gradients at 2.5 mM in experiment 1 or 5 mM in other experiments, and the gradient in experiment 2 also contained 2 mM reduced glutathione.

All gradients were centrifuged in a Beckman SW 25.2 rotor at 4° C in a L2 ultracentrifuge (Beckman Instruments, Palo Alto, Cal., USA) at 25,000 rpm (106,900 g) for 4.5 h, or 5 h in the case of experiment 1, after slow acceleration as described by Hanks et al. (1981). After the run, 2- to 5-ml fractions were collected from the top of the gradient.

Enzyme assays. Glutamine synthetase (EC 6.3.1.2) was assayed using the glutamyl hydroxamate synthetic assay according to Farnden and Robertson (1980). Glutamate synthase (EC 1.4.1.14) was assayed by measuring oxoglutarate and glutamine-dependent NADH disappearance as described by Boland and Benny (1977), and aspartate aminotransferase (EC 2.6.1.1) with the malate dehydrogenase-linked assay of Bergmeyer and Bernt (1963). Asparagine synthetase (EC 6.3.1.1) was assayed by the synthesis of [4-^{14}C]asparagine from labeled aspartate, followed by separation by paper electrophoresis according to Farnden and Robertson (1980).

Phosphoribosylpyrophosphate synthetase was assayed as described by Schubert (1981); phosphoribosyl amidotransferase was assayed using the PRPP-dependent deamidation of [^{14}C]glutamine, with subsequent separation of labeled glutamate by paper electrophoresis according to Holmes et al. (1973). Xanthine dehydrogenase was assayed as described by Boland (1981).

Phosphoglycerate dehydrogenase was assayed by measuring the phosphohydroxypyruvate-dependent oxidation of NADH at 340 nm. Reaction mixtures contained 0.1 M potassium phosphate buffer, pH 7.5, 60 μM NADH and 25 μl of a gradient fraction in a volume of 0.95 ml. The reaction was started by the addition of 50 μl of 20 mM phosphohydroxypyruvate. Serine hydroxymethylase was assayed by a modification of the method of Taylor and Weissbach (1965). The reaction mixture contained 80 mM potassium phosphate buffer, pH 7.5, 10 μg/ml pyridoxal phosphate, 0.2% (v/v) β-mercaptoethanol, 1 mg/ml FH_4 and 25 μl of a gradient fraction. After preincubation for 10 min, the reaction was started by the addition of serine to 0.8 mM, 20 kBq [3-^{14}C]serine per reaction in a total volume of 0.4 ml. After 20 min incubation, derivatization and extraction were carried out as described by Taylor and Weissbach (1965). Methylene FH_4 dehydrogenase was assayed by monitoring the increase in absorbance at 340 nm due to formation of NADPH and methenyl FH_4. A combined extinction coefficient of 30,000 M^{-1} cm^{-1} was used to calculate rates. Reaction mixtures contained 50 mM tris-(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 2.5 mM dithiothreitol, 400 μM formaldehyde, 400 μM FH_4 and 25 μl of a gradient fraction. After 10 min preincubation, the reaction was started by the addition of NADP$^+$ to give a final concentration of 50 μM.

Marker enzymes were assayed as follows. Triosephosphate isomerase (EC 5.3.1.1), a proplastid marker, was determined by coupling with α-glycerophosphate dehydrogenase according to Beisenherz (1955). Fumarase (EC 4.2.1.2), a marker enzyme for the mitochondria, was assayed in 0.1 M potassium phosphate, pH 7.5, by the production of fumarate, measured at 240 nm, in the presence of 50 mM malate (Biochemica Information[1]), and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) in the bacteroids was measured by 3-hydroxybutyrate dependent NAD$^+$ reduction as described by Hanks et al. (1981).

Gel electrophoresis. Gel electrophoresis and activity staining of aspartate aminotransferase was carried out in tube gels according to Reynolds and Farnden (1979).

Results and discussion

The gradient centrifugation effected separation of mitochondria, proplastid and bacteroid fractions as indicated by the marker enzymes, fumarase, triosephosphate isomerase and β-hydroxybutyrate dehydrogenase, respectively (Fig. 1). These organelles were found at densities of 1.18, 1.21 and 1.23 g ml^{-1}, respectively. A value of 1.21 g ml^{-1} has been reported for proplastids from spinach and pea roots by Miflin (1974). The presence of triosephosphate isomerase at the top of the gradient in the soluble fraction is due, at least in part, to proplastid breakage.

Enzymes synthesizing dicarboxylic amino acids and amides. Glutamine synthetase, the first enzyme responsible for assimilation of ammonia produced by

[1] Vol. II, p. 71; Boehringer, Mannheim, F.R.G.

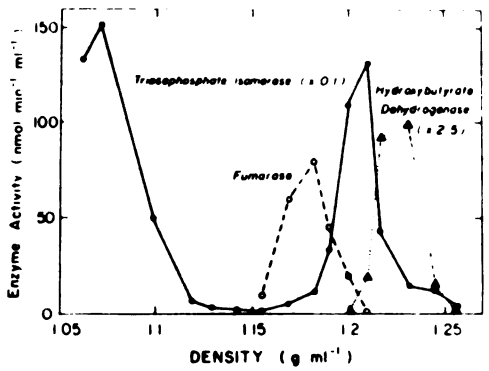

Fig. 1. Distribution of marker enzymes for mitochondria, proplastids, and bacteroids of soybean nodules following sucrose density gradient centrifugation. Triosephosphate isomerase × 0.1 (proplastid marker) (●), fumarase (mitochondrial marker) (○), and β-hydroxybutyrate dehydrogenase × 2.5 (bacteroid marker) (▲). Results shown are from expt. 3.

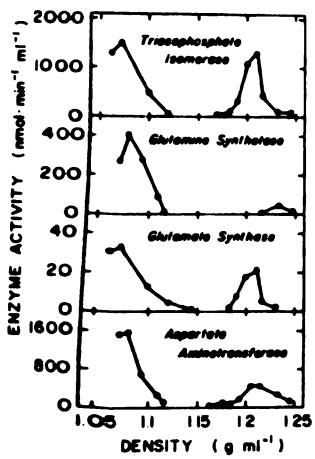

Fig. 2. Distribution of soybean nodule enzymes responsible for the synthesis of amino acids and amides after sucrose density gradient centrifugation. Triosephosphate isomerase and glutamate synthase results are from expt. 3; glutamine synthetase and aspartate aminotransferase results from expt. 1.

bacteroids in the nodule, was found almost entirely in the soluble fraction of the nodule (Fig. 2). This result is in agreement with the distribution of this enzyme in nodules of *Phaseolus vulgaris* reported by Awonaike et al. (1981) and in roots of maize, rice, bean, pea, and barley reported by Suzuki et al. (1981). Because of the unique role of glutamine synthetase in assimilating ammonium produced by the bacteroids, a cytosolic location seems a logical way of preventing a toxic buildup of ammonium.

The location of glutamate synthase in the cell was

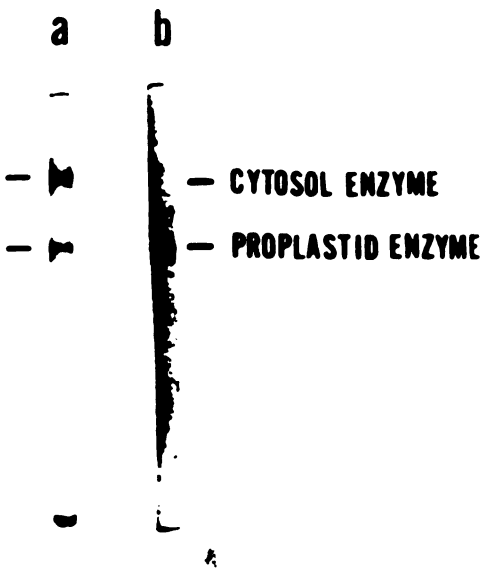

Fig. 3. Gel electrophoresis of soluble (a) and proplastid (b) fractions from soybean nodules, staining for aspartate aminotransferase activity.

not clear-cut; however, there was a definite peak of enzyme activity associated with the proplastid fraction. A proplastid location has been reported for glutamate synthase in *Phaseolus vulgaris* nodules (Awonaike et al. 1981) and soybean root tissue (Suzuki et al. 1981), and our results support this localization, although the possibility of an additional location in the cytosol can not be excluded.

Aspartate aminotransferase activity was found in both soluble and proplastid fractions (Fig. 2), in agreement with the results of Miflin (1974). The existence of two isoenzymes of aspartate aminotransferase has been reported for lupin nodules by Reynolds and Farnden (1979) and for soybean nodules by Ryan et al. (1972). The faster-moving lupin enzyme in gel electrophoresis is produced in the nodule concurrently with other ammonium assimilating enzymes during nodule development, and is presumably the primary enzyme responsible for aspartate synthesis in ammonia assimilation (Reynolds and Farnden 1979).

Following separation by gradient centrifugation, soluble and proplastid fractions were subjected to electrophoresis on tube gels and stained for aspartate aminotransferase activity (Fig. 3). The proplastids contained only one isoenzyme, while the soluble fraction contained two, the major one of which had a lower relative mobility than the proplastid isoenzyme. The second isoenzyme in the soluble fraction co-mi-

Table 1. Distribution of enzyme activities between soluble and peak mitochondrial and proplastid fractions from soybean nodules[a]

Fraction	Asparagine synthetase	PRPP synthetase	Phosphoribosyl amidotransferase
Soluble	0.21	3.1	2.8
Mitochondrial	ND	0.38	ND
Proplastids	0.31	0.38	4.0

[a] Values are from experiment No. 2. All values are expressed in nkat ml^{-1} gradient fraction. ND = none detected

grated with the proplastid isoenzyme and was probably the result of proplastid breakage. If a comparison with lupins is valid, then the faster-migrating, proplastid isoenzyme is specifically involved in aspartate synthesis during ammonia assimilation. Scans of the gels indicated that in the soluble fraction, the slower-moving isoenzyme accounted for approximately 70% of the total activity.

Asparagine synthetase, although not involved in ureide synthesis, is an important enzyme in amide-synthesizing legume nodules, and appears to be important in soybean nodules during a developmental phase prior to ureide production (Reynolds et al. 1982a). Because of the low levels of this enzyme in the soybean nodule and the difficulties of assay, only the peak fraction from each organelle band was assayed. The results are presented in Table 1. The enzyme was clearly located in the proplastids.

The overall distribution of this group of enzymes suggests that glutamine is synthesized in the cytosol, while synthesis of glutamate, aspartate and asparagine occurs in the proplastids, and possibly also in the cytosol.

Purine-synthesizing enzymes. Only two enzymes involved directly in purine synthesis have been successfully assayed in cell-free nodule extracts. PRPP synthetase catalyses the reaction immediately prior to the first committed step of the purine synthesis pathway. The majority of the enzyme activity was in the soluble fraction, although small amounts were associated with mitochondrial, proplastid and bacteroid fractions (Table 1), possibly because of nonspecific binding to these organelles. This occurrence in all organelle fractions is distinct from the case of proplastid enzymes, which were not found in significant quantities in other organelle fractions. The enzyme, however, is highly labile and a large proportion of the activity was lost during the centrifugation.

Phosphoribosyl amidotransferase catalyzes the first committed step of purine biosynthesis. As with asparagine synthetase, this enzyme was assayed in peak organelle fractions only. It was clearly located in the proplastids (Table 1), and also in the soluble

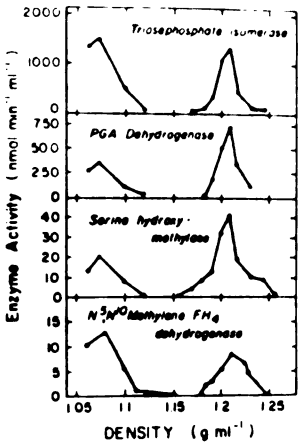

Fig. 4. Distribution of soybean nodule enzymes of the glycine C_1 pathway on a sucrose density gradient. Results for methylene FH_4 dehydrogenase were taken from expt. 1, all other activities are from expt. 3. *PGA* = 3-phosphoglycerate

fraction, although the activity in the soluble fraction is probably from broken proplastids.

Enzymes of glycine and methenyl-FH_4 synthesis. In plants, there are several alternative pathways for the synthesis of glycine. Recent studies with nodules (Reynolds et al. 1982a, b) indicate that glycine, along with methenyl-FH_4, is synthesized from serine which in turn is synthesized from the glycolytic intermediate, phosphoglycerate, by a series of reactions initiated by phosphoglycerate dehydrogenase. The gradient profile of this enzyme is presented in Fig. 4. There was a large proportion of activity in the proplastid fraction and lesser amounts of activity in the soluble fraction. The latter activity was attributed to proplastid breakage.

A similar distribution in proplastids was found for serine hydroxymethylase and methylene-FH_4 dehydrogenase (Fig. 4). Thus, it seems likely that the entire pathway from 3-phosphoglycerate to glycine plus methenyl-FH_4 occurs in the proplastids, where the products, glycine and N^5,N^{10}-methenyl FH_4, are conveniently available for a proplastid-based purine biosynthesis. The presence of serine hydroxymethylase in plastids contrasts with the location of this enzyme in leaves (Woo 1979; Tolbert 1981) where serine hydroxymethylase is in the mitochondria.

Recoveries of enzymes in proplastid fractions. The proportions of enzymes recovered in proplastid fractions from three different gradients are presented in Table 2. In the calculation of recoveries of phosphoribosyl amidotransferase and asparagine synthetase, only the two main fractions of the proplastid band

Table 2. Recovery of enzymes in the proplastid fractions of soybean nodules from three experiments

Enzyme	1	2	3
Triosephosphate isomerase	31	8.2	26
Glutamine synthetase	0	1.4	
Xanthine dehydrogenase			0
Phosphoglycerate dehydrogenase	62	29	46
Serine hydroxymethylase	55		41
Methylene FH_4 dehydrogenase		24	
Asparagine synthetase[a]		37	
Phosphoribosyl amidotransferase[a]		31	
Aspartate aminotransferase	21	9	
Plastid isoenzyme[a, b]	43		
PRPP synthetase		11	
Glutamate synthase	19	9.4	23

[a] For methods of calculation see text

[b] Of aspartate aminotransferase

were taken into account for the proplastid fraction, and it was assumed that the activities in the two soluble fractions were the same, although only one was assayed. All other fractions were treated as having no activity. For all other enzymes, all fractions were taken into account and the four peak fractions of the proplastid band were considered to constitute that band. (Each proplastid fraction contained 2 ml of a 58-ml gradient.) It is clear that xanthine dehydrogenase and glutamine synthetase were not located in the proplastids. The location of PRPP synthetase is uncertain. Of the enzymes found in the proplastids, there appear to be two classes: those which were probably located exclusively in the proplastids, for which relatively high and comparable recoveries were obtained in each experiment, and those for which a lower proportion of the total activity was found in the proplastid fraction.

The enzymes which are probably found only in the proplastids include those of the glycine-C_1 pathway, the proplastid isoenzyme of aspartate aminotransferase, asparagine synthetase and phosphoribosyl amidotransferase. To the group with lower recoveries in the proplastid fraction belong triosephosphate isomerase, total aspartate aminotransferase and glutamate synthase. Miflin (1974) has reported a similar result for triosephosphate isomerase in pea root tissue, and has suggested the possibility of both soluble and proplastid forms of the enzyme. Our results for the distribution of total aspartate aminotransferase activity are consistent with this explanation. Another possibility is that there might be a second, more easily broken class of proplastids, or an outer membrane on the proplastids which is more easily broken, selectively releasing these enzymes from the plastids. In view of the evidence in Table 2, phosphoglycerate dehydrogenase is a better marker enzyme for proplastids from soybean nodules than triosephosphate isomerase.

Proposed model of cellular organization of nitrogen assimilation in soybean nodules. In Fig. 5 we present a model for the subcellular distribution of the enzymes of ammonium assimilation and ureide biogenesis based on these results and the work of Hanks et al. (1981). In this model, ammonium produced by the bacteroids is incorporated into glutamine in the cytosol surrounding the peribacteroid sac. Further metabolism, at least as far as a purine ribonucleotide, then occurs in the proplastids. The occurrence of the purine biosynthetic pathway in the proplastids is implied by the presence of the enzyme catalyzing the first committed step of the pathway, plus ready sources in the proplastid of glutamine, glycine, aspartate, methenyl FH_4 and presumably CO_2, the other precursors for purine biosynthesis. A proplastid location for this pathway could explain why incorporation of purine precursors into ureides by nodule slices has been low (Atkins et al. 1980).

Rowe et al. (1978) have reported the isolation of the enzymes of purine synthesis from pigeon liver as a multienzyme complex which includes phosphoribosyl amidotransferase and all of the enzymes leading to the synthesis of IMP. The localization of purine biosynthesis and reactions of glycine and C_1 metabolism in the proplastid presents an alternative mechanism for substrate channeling or possible stabilization of a multienzyme complex. There is some question as to the nature of the final product of purine synthesis in nodules, or how it is converted to xanthine (Boland 1981; Boland and Schubert 1982); however, the pathway almost certainly leads to a purine monophosphate. Economy would dictate that hydrolysis of the purine monophosphate should occur in the cytoplasm so that the ribose (or ribose-5-phosphate) can be re-used for PRPP synthesis, assuming this occurs only in the cytosol. Xanthine dehydrogenase has been shown by Hanks et al. (1981) to occur in the cytosol of nodule cells, and subsequent oxidation and hydrolysis then occurs in the peroxisomes and endoplasmic reticulum.

Soybean nodules contain two different types of cells – larger, bacteroid-containing cells and smaller, uninfected cells. Newcomb and Tandon (1981) have reported that during cell development, the infected cells contain greatly increased numbers of mitochondria and proplastids which become crowded around the periphery of the cell. In contrast, in uninfected cells a large number of peroxisomes are present, together with increased smooth endoplasmic reticulum. Since uricase exists in the peroxisomes and allantoinase in the microsomal fraction resulting from the en-

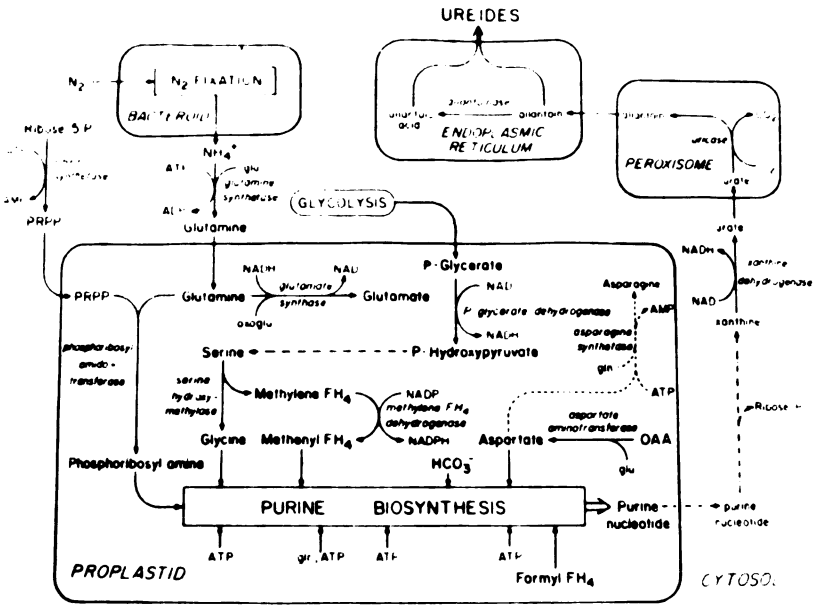

Fig. 5. Proposed model for the subcellular distribution of the enzymes of ammonium assimilation and ureide biogenesis in soybean nodules. ——, known enzymes; - - - -, reactions yet to be established; ····· reaction which occurs mostly in developing nodules. *OAA* oxalacetate, *oxoglu* 2-oxoglutarate, *gln* glutamine, *glu* glutamate

doplasmic reticulum (Hanks et al. 1981) it has been proposed by Newcomb and Tandon (1981) that at least the two steps of purine breakdown catalyzed by these enzymes are carried out in the uninfected cells. Since a large proportion of the proplastids are in the peripheral portion of the infected cells, it is possible that purine biosynthesis occurs in these cells. Future experiments will address the question of the distribution of the enzymes and organelles of ureide biosynthesis between infected and uninfected cells of the nodule, and further clarification of the function of nodule proplastids.

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