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ULTRASTRUCTURAL DISTRIBUTION OF GLUTAMATE DEHYDROGENASES DURING FRUIT BODY DEVELOPMENT IN *COPRINUS CINEREUS*

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Summary

Techniques based on a copper ferricyanide method were developed for the cytochemical localization at the ultrastructural level of NAD- and NADP-linked glutamate dehydrogenases (NAD–GDH, NADP–GDH), the latter enzyme being specifically involved in mushroom cap development. These techniques were validated using malate, lactate and succinate dehydrogenases (MDH, LDH, SDH). Tests were applied to unfixed and formaldehyde-fixed material from three developmental stages of fruit bodies of *Coprinus cinereus* (Schaeff.: Fr.) S.F. Gray *sensu* Konr. (1) before spore formation (stage 2), (2) with young spores (stage 3), and (3) with mature spores (stage 4). Spectrophotometric assay showed that NADP–GDH was still detectable after 15 min formaldehyde fixation and approximately 9 and 80% activity of NAD–GDH and MDH, respectively, was preserved. At all stages of development MDH, LDH and SDH activities were localized in mitochondria or mitochondrion-like bodies. In contrast, both NAD–GDH and, especially NADP–GDH, were localized in cytoplasmic vesicles, mainly in basidia and very young spores, sometimes being found adjacent to the cell walls, suggesting that these enzymes are transported to the vicinity of the plasma membrane. Such a location would be consistent with enzymological data, indicating that NADP–GDH contributes to an ammonium scavenging system. Ultrastructural localization of this system at the peripheral region of basidia suggests it may be linked to the synthesis and/or assembly of cell wall components and may protect the meiotic apparatus against ammonium inhibition.

Key words: *Coprinus*, glutamate dehydrogenase, cytochemistry, ultrastructural, localization.

Introduction

Coprinus cinereus (Schaeff.: Fr.) S.F. Gray *sensu* Konr. possesses two types of glutamate dehydrogenase (GDH), linked to the co-enzymes NAD (NAD–GDH) and NADP (NADP–GDH), respectively (Fawole & Casselton, 1972; Stewart & Moore, 1974). Analysis of cell-free extracts has indicated that activity of both enzymes increases during fruit body development, although while NAD–GDH activity rises in both cap and stipe, NADP–GDH activity increases only in the gill tissues of the cap (Stewart & Moore, 1974; Ewaze, Moore & Stewart, 1978). This cap-specific developmental control of NADP–GDH is of interest as a model of morphogenetic regulation in higher fungi. The enzymes have been localized cytochemically in frozen sections at the light microscope level by means of tetrazolium staining (Elhiti, Butler & Moore, 1979). In caps, the NAD–GDH was at first distributed uniformly in the basidia, but an increasing number of subhymenial cells showed activity as the fruit body matured. The NADP–GDH

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was limited initially to isolated patches of basidia, but later the proportion of basidia and subhymenial cells showing activity increased progressively.

Analyses of other enzyme activities, and metabolite levels (reviewed in Moore, 1984) indicate that development of the fruit body cap requires amplification of tricarboxylic acid cycle (TCA) and urea cycle activity, the net result being accumulation of urea, and probably other nitrogenous metabolites, as osmotic solutes which drive water into the cells of the gill hymenium. The influx of water leads to inflation of these cells, and their expansion can account for the changes in form through which the cap progresses as maturation proceeds (Moore, Elhiti & Butler, 1979). Elevated activity of NADP-linked GDH (which is highly positively correlated with increase in glutamine synthetase activity) has been interpreted as representing co-ordinated derepression of an ammonium scavenging system which safeguards the operation of the TCA cycle. In *C. cinereus*, the TCA cycle is linked with a 4-aminobutyrate decarboxylation loop which starts with amination of 2-oxoglutarate (Ewaze *et al.*, 1978). This suggestion has received some support from ultrastructural observations showing that NADP–GDH activity has a peripheral location in mycelia subjected to nitrogen starvation (Elhiti, Moore & Butler, 1986).

This paper describes the ultrastructural localization of dehydrogenases during fruit body development. Techniques based on the copper ferricyanide method (Ogawa, Saito & Mayahara, 1968; Kerpel-Fronius & Hajos, 1968) were elaborated for the localization of NAD–GDH and NADP–GDH in fixed and unfixed tissues. These were validated using malate dehydrogenase (MDH), lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH).

MATERIALS AND METHODS

Fungal isolate used

The BC/6,6 × Hl dikaryon of *C. cinereus* (Schaeff.: Fr.) S.F. Gray *sensu* Konr. (= ATCC 42725 × ATCC 18065) was used to produce fruit bodies by growing it on sterilized horse dung (Moore & Ewaze, 1976). Three of the five developmental stages (Moore *et al.*, 1979) have been used for these studies: stage 2, immature fruit body before spore formation; stage 3, fruit body with young spores; stage 4, fruit body with mature spores.

Cytochemistry

Trials confirmed that glutaraldehyde is ineffective in preserving dehydrogenase activity (see below). Buffered formaldehyde solution 'D' (Carson, Martin & Lynn, 1973) gave the best compromise between preservation of ultrastructure and enzyme activity. This consists of 10% (v/v) technical grade formalin (= about 4% formaldehyde) containing 0·1 M NaOH and 0·1 M NaH_2PO_4.

No GDH activity could be detected with thiocarbamyl nitroblue tetrazolium (Seligman *et al.*, 1968) or tetranitroblue tetrazolium (Ogawa & Barrnett, 1965), but a method was developed from that of Ogawa *et al.* (1968) which was satisfactory for all dehydrogenases, using ferricyanide as an electron acceptor which, on reduction, is converted into insoluble cupric ferrocyanide. This method has been used previously to demonstrate several dehydrogenases and oxidases in both plant (Bell, 1979) and animal (Sasaki, 1979) cell types (for a review, see Borgers & Verheyen, 1985). The ferricyanide method allows demonstration of enzymatic activity in fresh incubated tissues with little of the morphological damage evident with other methods.

For tests on prefixed material, tissue was fixed in buffered formaldehyde solution 'D' (Carson *et al.*, 1973) for 20 to 25 min at 4 °C, washed in 0·1 M phosphate buffer (pH 7·2) containing 0·02 M sucrose and incubated in copper ferricyanide medium for 90 min at 37 °C, buffer-washed, post-fixed in buffered 2% osmium tetroxide for 1 h at 40 °C, dehydrated in ethanol and embedded in epoxy resin (Spurr, 1969).

The standard ferricyanide mixture contained (in a total of 10 ml): 6·0 ml sodium potassium tartrate (0·5 M), 1·6 ml phosphate buffer at pH 7·2 (0·1 M), 0·7 ml copper sulphate (0·3 M) added dropwise and the pH of the mixture adjusted to 6·6 to 6·8. Dimethyl sulphoxide (DMSO) (1·4 ml) was added as an intermediate electron acceptor for MDH and LDH only, being replaced with buffer in other reactions, and the solution was completed by dropwise addition of 0·3 ml of 0·05 M potassium ferricyanide. Substrates and co-enzymes were included as follows: GDH, L-glutamic acid (monosodium salt, 0·125 M) and 10 mg NADP; MDH, L-malic acid (0·06 M) and 5 mg NAD; LDH, DL-lactic acid (0·025 M) and 5 mg NAD; SDH, sodium succinate (0·25 M) only. Sodium azide was included as a respiratory chain inhibitor for MDH (0·1 ml of 0·1 M solution per 1·0 ml of medium), and sucrose (0·02 M final concentration) was added to all incubation media except those for SDH. All tests included controls lacking the substrate and an unincubated blank.

For tests on unfixed fresh tissues, samples of caps and stipes were incubated in the appropriate reaction mixture, washed in buffer, fixed in 3% buffered glutaraldehyde for 2 h, post-fixed for 2 h in 2% buffered osmium tetroxide and embedded in epoxy resin.

X-ray micro-analysis

Identification of the electron-dense end-product of all cytochemical tests was confirmed by X-ray micro-analysis, copper and iron being used as markers of the end-product, cupric ferrocyanide. Thick (200 to 250 μm) sections were mounted on 400 mesh formvar–carbon–coated aluminium grids, coated with carbon and examined in an AEI CORA analytical transmission electron microscope using a beam current within the range of 1 to 2×10^7 A on 60 kV accelerating voltage, a 250 to 400 nm spot size and a count time of 100 s.

Spectrophotometric analysis

The effects of chemical fixation on enzyme activity were determined using spectrophotometric assays of cell-free extracts of stage 4 cap tissues. Both GDH enzymes were assayed in the amination direction (Al-Gharawi & Moore, 1974), and MDH activity was assayed as described by Moore & Ewaze (1976). Sliced tissues were divided into 200 mg batches which were fixed separately at 4 °C for 30 min in 3% glutaraldehyde in phosphate buffer (pH 7·2), or for 15 or 30 min in Carson's formaldehyde solution 'D' fixative (Carson *et al.*, 1973); other batches remained unfixed. Subsequently, the samples were washed, then homogenized in buffer (3 ml per 200 mg of tissue), and the filtrates of the homogenates assayed for GDH and MDH activities.

The possibility that NADP–GDH activity is associated with cell walls (see below) was examined by using the same spectrophotometric enzyme assays to measure enzyme activity of: (1) suspensions of mature and germinated intact spores, (2) freeze-thawed cap tissue, and (3) the filtrate and debris of cell-free extracts prepared by homogenization of fresh cap tissue.

RESULTS

Chemical fixation and enzyme activity

After 30 min of glutaraldehyde fixation, all spectrophotometrically detectable enzyme activity was lost. After 30 min fixation in formaldehyde, the NADP-GDH activity was lost, and that of NAD–GDH barely detectable but more than 50% of the MDH activity was preserved. With 15 min formaldehyde fixation, some NADP–GDH activity was detectable, while about 9% of NAD–GDH and 80% of MDH activity was preserved (Table 1). These data were used as a guide for designing and interpreting cytochemical tests.

Table 1. *Residual activities of NAD–GDH, NADP–GDH and MDH enzymes in cell-free homogenates of early stage 4 fruit body cap tissues of* Coprinus cinereus *subjected to fixation*

Treatment	Enzyme activity remaining (%)		
	NADP–GDH	NAD–GDH	MDH
Glutaraldehyde (30 min)	0	0	0
Formaldehyde (30 min)	0	1	65·6
Formaldehyde (15 min)	2·4	9·2	80·0

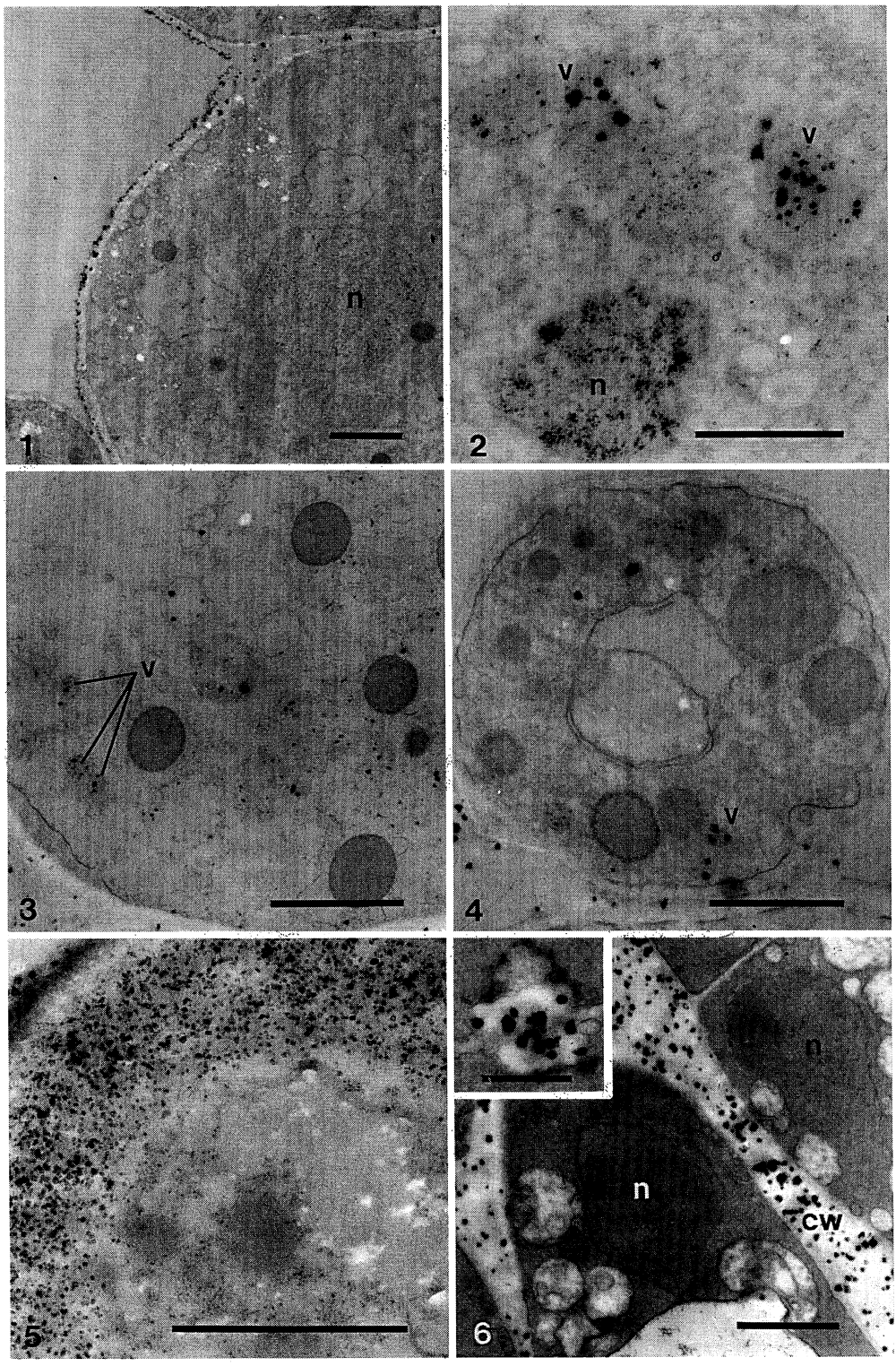
Cytochemistry

NAD-specific glutamate dehydrogenase (*NAD–GDH*). In tests with 20 to 25 min formaldehyde-prefixed stage 3 tissues, the major reaction was in the nuclei, most of which was lacking in controls (Figs 1 and 2). Free particles were seen also, within small (120 to 230 nm) cytoplasmic vesicles and outside the cells (Fig. 3). In young spores, reaction deposits were present in the cytoplasmic ground substance and in small cytoplasmic vesicles (Fig. 4) and, in older spores, in association with the cell walls (Fig. 5). No deposits were found in the blank, unincubated samples.

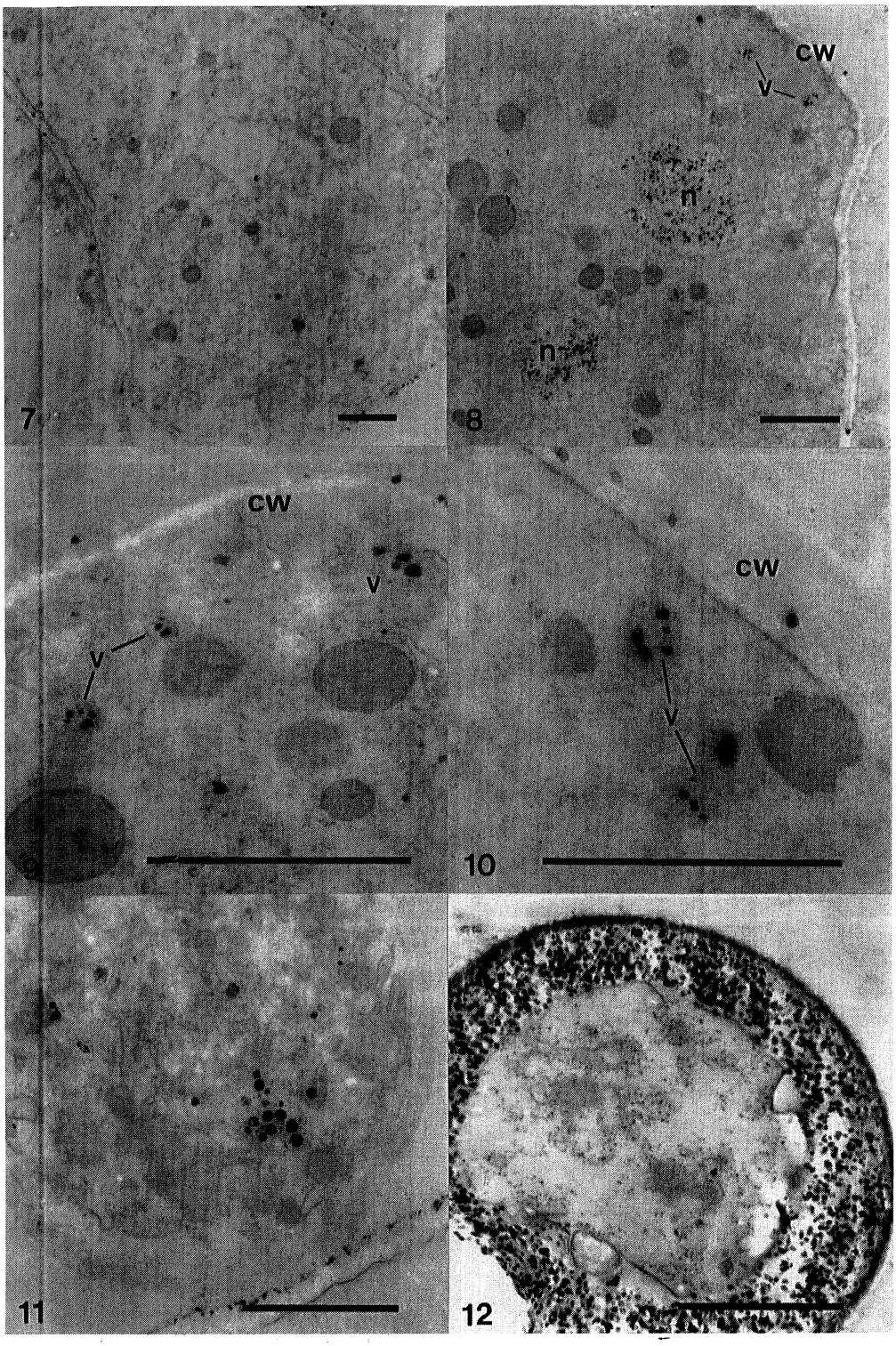
In unfixed samples of stage 2 cap material, deposits were seen in association with the cell wall and dolipore septa, and, in some cells, nuclei and cytoplasmic vesicles (Fig. 6). In unfixed stage 3 caps, basidia contained dense deposits in unidentified membranous structures.

NADP-specific glutamate dehydrogenase (*NADP–GDH*). In controls of early stage 3 prefixed cap tissue, a few deposits were seen (Fig. 7) but they were absent from the blank unincubated samples. In tests, most deposits were found inside infrequent 120 to 230 nm vesicles located mainly in basidial cells and very young spores, adjacent to the cell walls. Occasionally, deposits were seen also free in the

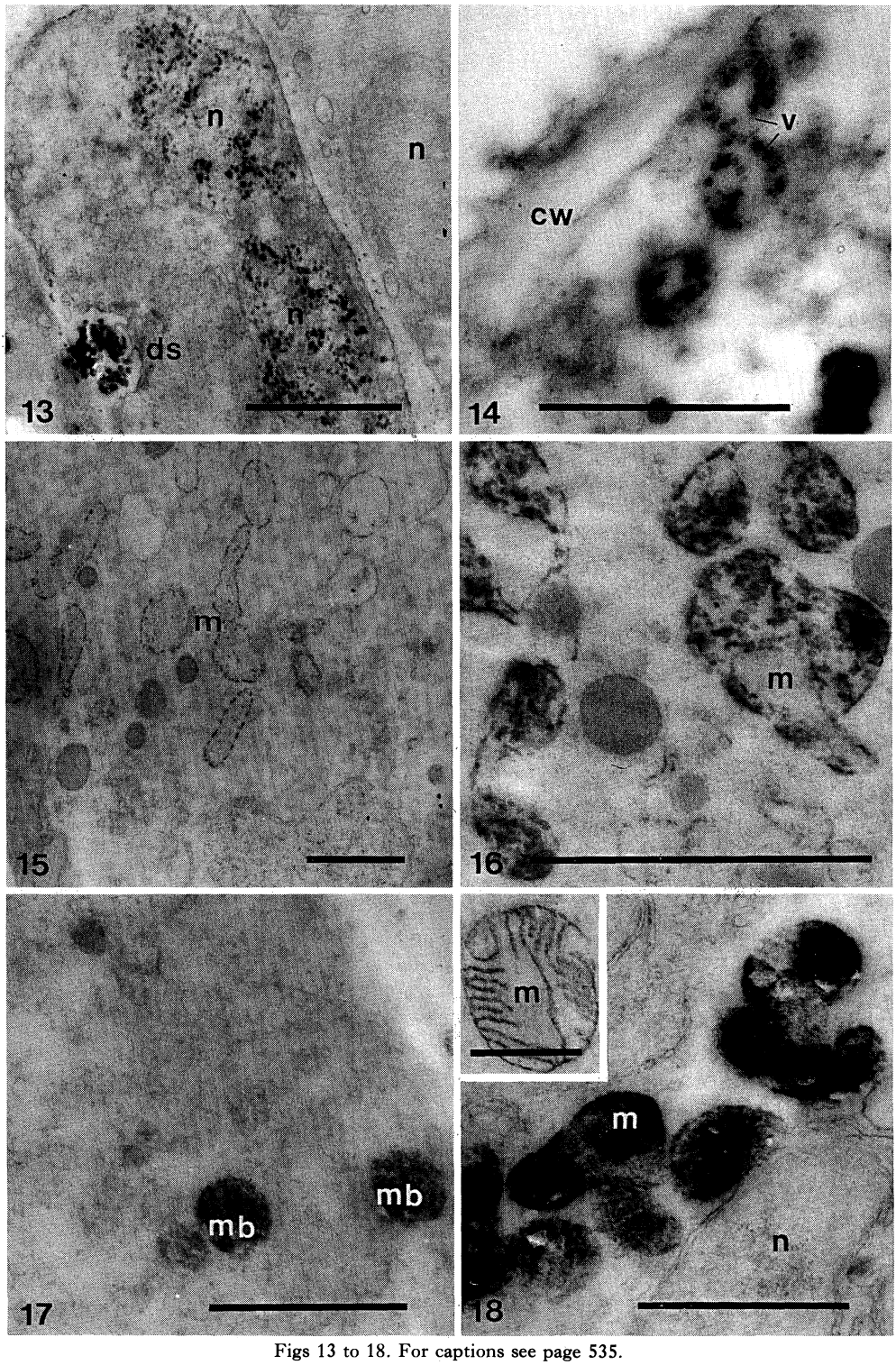
Figs 1 to 6. TEM sections of copper ferricyanide tests for NAD-linked glutamate dehydrogenase in *Coprinus cinereus*. Figures 1 to 5, prefixed, stage 3; Figure 6, unfixed, stage 2. Fig. 1 shows the control of fruit body cap tissue, a few fine deposits occur only in the nuclei (n), and on the outer wall (bar = 1·0 μm; × 10 500). Figs 2 and 3, test of gill tissue with deposits of reaction product in nuclei (n), cytoplasmic vesicles (v), cytoplasmic ground substance and outside the cells (bar = 1·0 μm; Fig. 2 × 21 950; Fig. 3 × 19 500). Fig. 4, young spore with reaction product deposit in cytoplasmic ground substance and vesicles (v) (bar = 1·0 μm; × 19 800). Fig. 5, old spore with reaction deposit associated with cell walls (bar = 1·0 μ; × 34 850). Fig. 6, cells of fruit body cap with deposit in cell wall (cw), and dolipore septa (inset), but not in nuclei (n) (bar = 1·0 μm; main figure × 14 650, inset × 26 000).



Figs 1 to 6. For captions see page 534.



Figs 7 to 12. For captions see page 535.



Figs 13 to 18. For captions see page 535.

cytoplasm, within nuclei or in intercellular spaces (Figs 8 to 10). In late stage 3 material, most of the reaction product appeared in association with the spore wall, although a finer deposit was sometimes found in cytoplasmic vesicles. No wall deposits were found in the controls (compare Figs 11 and 12). In other cells of the gill tissue, deposits were found occasionally in cytoplasmic vesicles and nuclei. Some deposits were seen in controls but none in the blanks.

Unfixed stage 2 material showed deposits in nuclei, dolipore septa and occasionally inside small cytoplasmic vesicles (Fig. 13). At stage 3, the enzyme distribution was similar to that found for NAD–GDH. Most deposits were found in cytoplasmic vesicles, sometimes adjacent to the cell walls (Fig. 14) and in dilated mitochondria. In unfixed material at stage 4, deposits were in the cytoplasm or in close association with the cell walls, with a little in cytoplasmic vesicles. No deposit was found in the spores except for a scattering outside the wall.

Malate dehydrogenase (*MDH*). In prefixed stage 3 cap tissues, reaction deposit was seen in the mitochondria of the hymenium, subhymenium, trama layer and cystidia. Most mitochondria showed a uniform distribution of deposit in the outer compartment and in the intracristal space (Figs 15 and 16), but deposits sometimes covered the whole mitochondrion whilst other mitochondria showed little deposit. Small amounts of background deposit could be seen outside the cells, around the vacuoles, in the cytoplasm or in the nuclei. X-ray microanalysis confirmed that the mitochondrial *and* extracellular deposits contained iron and copper. The extracellular deposit was also present in controls incubated without substrate. In stage 4 material, there was a similarly distributed positive reaction in mitochondria of basidia, paraphyses, cystidia and spores.

Lactate dehydrogenase (*LDH*). In stage 3 and 4 samples prefixed for 15 min, the reaction was restricted to bodies with mitochondrion-like profiles but variable in both size and activity level (Fig. 17). Slight deposits occurred within the spore walls. This was also seen in the controls but there was no intracellular reaction.

Figs 7 to 12. TEM sections of copper ferricyanide tests for NADP-linked glutamate dehydrogenase on prefixed stage 3 caps of *Coprinus cinereus*. Fig. 7 shows the control reaction for a basidium; few deposits are evident (bar = 1·0 μm; × 8300). Figs 8 to 10 show results of test reactions on basidia. Deposits of reaction products are located mainly in cytoplasmic vesicles (v), often in association with cell wall (cw). Deposits are also found in some nuclei (n), and the cytoplasmic ground substance (bars = 1·0 μm; Fig. 8 × 12000; Fig. 9 × 39600; Fig. 10 × 40450). Figs 11 and 12, spores showing a control with no deposits of reaction product in the wall (Fig. 11; bar = 1·0 μm; × 23150) and a test with such deposits in the wall (Fig. 12; bar = 1·0 μm; × 24450).

Figs 13 to 18. TEM sections of tests for NADP-linked glutamate dehydrogenase and other dehydrogenases on cap tissues of *Coprinus cinereus*. Figs 13 and 14, tests for NADP-linked glutamate dehydrogenase. Fig. 13, unfixed stage 2 cap. Deposits are present in nuclei (n) and dolipore septa (ds) (bar = 1·0 μm; × 23050). Fig. 14, unfixed stage 3 cap. Most deposits are in cytoplasmic vesicles (v), sometimes adjacent to the cell walls (cw) (bar = 1·0 μm; × 36800). Figs 15 and 16, prefixed stage 3, malate dehydrogenase. The control (Fig. 15; bar = 1·0 μm; × 14200) shows only slight deposits in the mitochondria (m), whereas the test (Fig. 16; bar = 1·0 μm; × 50000) shows heavy deposits in both the outer compartment and intracristal space of the mitochondria (m). Fig. 17, prefixed stage 4, lactate dehydrogenase. Various levels of reactivity are present in mitochondria-like bodies (mb) (bar = 1·0 μm; × 58400). Fig. 18, unfixed stage 3, succinate dehydrogenase. Most mitochondria (m), showed heavy deposits of reaction product, although in some the reaction was confined to the outer membrane at the surface of cristae (inset). The nuclei (n), show no deposit (main figure, bar = 1·0 μm; × 30700; inset, bar = 0·5 μm; × 32450).

Succinate dehydrogenase (*SDH*). Good localization of this enzyme was demonstrated only in freshly incubated samples in which a heavy mitochondrial deposit was found in both gill tissues and spores, although there was considerable heterogeneity (Fig. 18). No significant staining occurred in the nuclei. The controls had no mitochondrial deposits, although some showed staining of the outer membrane.

Spectrophotometric enzyme assays

No detectable activity of NADP–GDH was found in suspensions of intact spores. In tissue samples subjected to freezing and thawing, only 6·5 % of total activity was associated with the particulate material after a single freeze–thaw cycle (3 % after two washes). In tissue homogenates, an average total activity of 0·115 OD units min^{-1} was separated into a filtrate activity of 0·087 and debris activity of 0·015 OD units min^{-1} (unit volume)$^{-1}$, respectively, by a single filtration step (without washing).

DISCUSSION

Interpretation of these data must take into account the differential sensitivity of enzyme activity to fixation (Table 1). With NADP–GDH, only 2 % of its activity remained after fixation. Consequently, observations made on prefixed material must be accompanied by those made on freshly reacted samples despite the inevitably poor ultrastructural preservation in them. Another problem is the frequent observation of reaction product outside the tissue. The dehydrogenase reaction chain depends on reducing power which could be obtained from a number of sources independent of the enzyme under test. Thus, identification of substrate-dependent formation of reaction product is essential.

Support for the distribution of GDH enzymes demonstrated here is provided by the fact that the other dehydrogenases show unexceptional patterns, with localizations similar to those observed in other tissues (Table 2). The MDH is

Table 2. *Summary of enzyme locations in fruit body cap tissues of* Coprinus cinereus

Enzyme	Location	Comments
NADP–GDH	Cytoplasmic vesicles, mainly in basidia	Most probable location
	Nucleus	Possible artifact
	Cell wall	Artifact
NAD–GDH	Cytoplasmic vesicles	Most probable location
	Nucleus	Possible artifact
	Cell wall	Artifact
MDH	Mitochondria	All cells in gill tissue
SDH	Mitochondria	All cells in gill tissue
LDH	Small, mitochondrion-like bodies	—

localized in mitochondria and its activity increased from stage 3 to stage 4, an observation which parallels data from assays of cell-free homogenates (Ewaze *et al.*, 1978). SDH was also localized to mitochondria, and the distribution of MDH- and SDH-reactive mitochondria throughout all the cells of the hymenium and subhymenium indicates that all cells are involved in metabolism utilizing these

enzymes. The heterogeneity of the mitochondrial population for both of these enzyme activities has been observed before in fungi (Koke, Gupta & Malhotra, 1971) and in animal cells (Ogawa & Barrnett, 1965; Seligman *et al.*, 1968; Haydon *et al.*, 1968). Activity of LDH was demonstrated in small mitochondrion-like bodies of uncertain identity.

Cytochemical activity of both NAD–GDH and NADP–GDH was shared between nuclei, cytoplasmic vesicles and cell wall (in particular the spore wall). In fixed samples, NAD–GDH was located mainly in nuclei, whereas NADP–GDH activity occurred mostly in vesicles and spore walls. When unfixed samples were used, both enzymes were observed in each location at some stage of development.

The high wall-associated GDH activity implied by images such as Figures 5 and 12 is certainly an artifact. Bearing in mind the effect of fixation on GDH activity (98 % of NADP–GDH activity lost; Table 1), the density of apparent reaction–product deposition within spore (and hymenial) walls would imply an extremely high level of activity of NADP–GDH in the particulate fraction obtained when tissue is homogenized. Yet spectrophotometric enzyme assays showed that only 13 % of total activity was associated with (unwashed) particulate debris separated from homogenized cap tissue. We conclude that there is no appreciable activity of NADP–GDH associated with the walls. Deposits of reaction products in the wall may be due to an affinity of the wall (especially carboxylate and polyphosphate groups) for metal ion components of the reaction mixture. Metal ion binding is certainly a feature of fungal biomass, both dead and alive (Tobin, Cooper & Neufeld, 1984).

In animal cells, GDH activity has been demonstrated in nuclei and mitochondria (di Prisco, Banay-Schwartz & Strecker, 1968) and purification of enzyme from the two sites yielded proteins having some differences in antigenicity (Casola, Ruffilli & di Prisco, 1974). Mitochondrial locations were indicated for *Dictyostelium* and the ciliate *Tetrahymena* (Smith *et al.*, 1975), but, on the other hand, fungal GDH enzymes seem mainly to have been located in the soluble phase of the cytosol. Diffusion of the reaction product can lead to deposition of heavy metal ions at chemically favourable sites (which occur especially in nuclei) some distance from the true position of the enzyme (De Jong *et al.*, 1979a, b), and diffusion and adsorption of the soluble enzyme protein may occur (Hayat, 1973; van Duijn, 1974; Hanker, 1975). Thus, the nuclear location of GDH activity in *Coprinus* requires separate confirmation and may be artifactual.

The vesicular deposits remain a probable positive location of the GDH enzymes. The vesicle deposits were seen for both GDH enzymes but were particularly evident in the NADP–GDH tests and most emphasized in early stage 3 material. Some of the vesicles were seen in association with the plasmalemma and the cell wall. The occurrence of NADP–GDH within membrane-bound cytoplasmic vesicles in basidia suggests that the appearance of this enzyme activity involves not only *de novo* enzyme synthesis (Jabor & Moore, 1984) but construction of specific cytoplasmic organelles which may transport the enzyme from its site of synthesis to a final location at the plasmalemma, or periplasmic space. A rather similar distribution of NADP–GDH has been demonstrated in mycelia subjected to nitrogen starvation (Elhiti *et al.*, 1986). Such a peripheral location would be consistent with the idea that this enzyme contributes to an ammonium scavenging system (Ewaze *et al.*, 1978; Moore, 1984) in mycelial hypha and basidium alike, but the view that the scavenging system is metabolically devoted to assimilation of ammonium and safeguards operation of the tricarboxylic

acid cycle under conditions of ammonium starvation (Moore, 1984), while attractive in the case of mycelium, is difficult to reconcile with other features of the developing fruit body, particularly the probable translocation of nitrogenous metabolites (Blayney & Marchant, 1977).

NADP–GDH has been found mostly within vesicles, mostly within basidia. Vesicles in *C. cinereus* have been reported to be associated with the provision of cell wall materials to the developing basidiospore wall (McLaughlin, 1974), and vesicular chitosomes are part of the accepted mechanism of fungal wall biogenesis (Gooday & Trinci, 1980), so there may be an association between NADP–GDH and wall synthetic mechanisms. One possibility is contribution of amino groups to the synthesis of glucosamine and hence, chitin. However, study of osmotic bursting of hyphal tips in *Mucor rouxii* showed that NH_4OH solutions in excess of 50 mM were more able to cause bursting than KOH or NaOH solutions of like pH, implying activity of the NH_4^+ ion in disrupting wall synthesis (Bartnicki-Garcia & Lippman, 1972). Conversely, therefore, the primary function of the NADP–GDH/glutamine synthetase ammonium scavenger in *C. cinereus* may be to remove ammonium from the environment of some process which is sensitive to ammonium inhibition. As NADP–GDH has been localized to basidia – the meiocytes – the most relevant comparison may be with meiosis and sporulation in yeast, which are inhibited by ammonium (Piñon, 1977) and dependent on glutamine synthetase activity (Delavier–Klutchko *et al.*, 1980).

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