

Glycogen metabolism in relation to fruit body maturation in *Coprinus cinereus*

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During development of the fruit body of *Coprinus cinereus* large quantities of glycogen are accumulated in the cap and are subsequently degraded during basidiospore formation. The major increase of glycogen concentration was initiated as karyogamy became evident in normally developing fruit bodies and the onset of large-scale utilization of accumulated glycogen was a post-meiotic event. The temporal pattern of glycogen accumulation/degradation was very variable among 23 different fruit body caps when judged on a common developmental time scale. This indicates that glycogen content was not closely associated with meiosis or basidiospore formation even though glycogen degradation usually started from about the onset of the latter. Glycogen phosphorylase was the only glycogen degradation enzyme found in cap tissues. Glycogen synthase activity could be demonstrated (and increased with time) even during the phase of most rapid glycogen degradation. Analysis of glycogen contents during *in vitro* gill development and in sporeless, stem elongationless and cap expansionless mutants suggested that glycogen degradation was not specific for any one of these processes. Nutrient translocation occurred mainly in the 'stem to cap' direction and glycogen influx to the cap continued into the phase of glycogen degradation in the cap. The overall conclusion is that glycogen acts as a carbohydrate supply which cannot be linked exclusively, or even predominantly, with any one of the several processes which occur as the fruit body matures but rather serves any or all of those processes as required.

In the earliest stages of growth of the dikaryotic mycelium of *Coprinus cinereus* (Schaeff.: Fr.) S. F. Gray glycogen is accumulated in bulbous cells of the submerged mycelium (Madelin, 1960; Waters, Butler & Moore, 1975). Madelin (1960) showed that the mycelial accumulations disappeared at the time of fruit body formation, and Jirjis & Moore (1976) found that decline in the quantity of glycogen in the dikaryotic mycelia coincided with the appearance of mature sclerotia when temperature and light regimes prevented fruiting. The implication seems to be that glycogen formed by the vegetative mycelium is translocated towards sites at which multicellular structures are formed and that it accumulates in those structures to provide for their development. Accumulation of glycogen in fruit body tissue is evident at very early stages of development (Matthews & Niederpruem, 1973). It is initially deposited in the base of the stem and subsequently disappears from this location as accumulations grow in the cap (Moore, Elhiti & Butler, 1979) where it becomes concentrated in subhymenial tissues (McLaughlin, 1974). Each young fruit body may contain in excess of 1 mg (d.w.) of glycogen, all of which disappears as basidiospores are produced (Moore *et al.*, 1979). It appears likely that glycogen is used to provide for basidiospore formation and that there is an organized translocation route (from mycelium

to stem and then to cap) to supply the polysaccharide to the hymenium. However, neither of these propositions has been tested.

In *Saccharomyces cerevisiae* Hansen glycogen synthesis takes place in both sporulating and non-sporulating strains but only sporulating strains degrade the polysaccharide (Pontefract & Miller, 1962; Roth & Lusak, 1970; Kane & Roth, 1974; Clancy, Smith & Magee, 1982; Katohda *et al.*, 1988). Such a close linkage between glycogen degradation and sporulation in *Coprinus* is also feasible. Meiosis is highly synchronized in *C. cinereus*. Lu (1982) estimated that in a single fruit body of *C. cinereus* there were approximately 1 to 3×10^7 basidia, of which 70 to 75% were at the same stage of meiosis. However, there are many other developmental processes, such as basidiospore formation, stem elongation and cap expansion, that occur during fruit body maturation which have not been distinguished. Also, most studies have relied on aspects of fruit body morphology to describe the developmental stage of the specimens studied (Madelin, 1956; Takemaru & Kamada, 1972; Matthews & Niederpruem, 1973; Morimoto & Oda, 1973; Moore *et al.*, 1979). Pukkila, Yashar & Binninger (1984) introduced nuclear staining to define the stage of development of the whole fruit body in terms of progress through meiosis and basidiospore formation in the hymenium. The technique has been developed to provide an objective and reliable time base (Hammad *et al.*, 1993a). In this research we have studied glycogen metabolism in relation to such a

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time-course and in strains defective in different aspects of fruit body maturation and we have examined nutrient exchange between cap and stem.

MATERIALS AND METHODS

Cultures

One wild-type strain (the Meathop dikaryon) and three mutant strains were used. The latter all carried dominant developmental mutations (Takamaru & Kamada, 1972); NG 107 is a sporeless mutant, strain NG 0398 is a stem elongationless mutant and UV 487 is an expansionless mutant. The three mutant strains were kindly provided by Professor T. Kamada, University of Tokyo.

Cultivation

Cultures were grown on Yeast Extract–Malt Extract–Glucose (YMG) Medium (Moore & Pukkila, 1985) in 9 cm Petri dishes. Petri dish cultures were grown in the dark at 37 °C. Vegetative cultures of the dikaryon were maintained by serial transfer between Petri dishes. Fruit bodies were produced by inoculating sterilized horse dung with pieces of dikaryon taken from the Petri dish cultures. The dung cultures were incubated in the dark for 3 days at 37° to allow establishment of mycelium before transfer to a 27° incubator with a 16 h light/8 h dark photoperiod, in order to induce the production of fruit bodies. Illumination was provided by white fluorescent lights which gave an average illuminance of 800 lx.

Determination of the time-course of meiosis and sporulation

Meiotic division stages were examined by using the silver staining technique described by Pukkila & Lu (1985). Sporulation stages, such as appearance of sterigmata, basidiospore formation and pigmentation, were visible without staining. Samples of a few gills were removed from the cap at 0.5 to 1 h intervals during meiosis and every 2 h before and after meiosis. The tissue was fixed in 4% (w/v) formaldehyde for 10 minutes at room temperature. A small piece (*ca* 1 mm²) of a single layer of basidia was removed using a pair of fine tweezers and transferred to a drop of 45% (v/v) acetic acid on a coverslip. The gill segment was torn into several pieces, squashed and stained (Pukkila & Lu, 1985). Using phase contrast optics, over 100 basidia were counted in each sample and the stage of development of each recorded. The proportions of the different developmental stages in each specimen were calculated and referred to a 'calibration curve' to standardize their developmental timing (Hammad *et al.*, 1993*a*). Glycogen metabolism during sporulation *in vitro* was studied in gill segments explanted to agar-solidified buffer medium as described by Chiu & Moore (1988*a, b*).

Measurement of nutrient exchange between cap and stem

To obtain ¹⁴C labelled caps and stems, cultures were fruited on YMG agar and 1 µCi D-[U-¹⁴C]glucose was injected into the

agar when primordia were first observed (before karyogamy). The stage when the stem started elongation (about 11 h after karyogamy), was chosen for grafting experiments. A graft consisted of either a labelled cap put onto an unlabelled stem or an unlabelled cap put onto a labelled stem. To measure physical diffusion artificial caps and stems were made from filter paper and substituted for the unlabelled cap or stem, respectively. Grafted fruit bodies were incubated for 4 h at 26°. Tissues were homogenized in a Griffiths type glass homogenizer in 2 ml distilled water. The homogenate was then transferred to an Eppendorf tube and centrifuged in a Microfuge at 13 000 rpm for 15 min. The supernatant was poured into scintillation vials containing 10 ml of Optiphase Hisafe II scintillation fluid for scintillation counting.

Glycogen determination

The glycogen content of fruit body tissues was determined using the iodine method of Krisman (1962) as modified by Jirjis & Moore (1976). Glycogen was also determined according to Gunja-Smith, Patil & Smith (1977) by measuring the glucose released by α-amylase and amyloglucosidase digestion. The glucose was determined by the glucose oxidase method using a commercial kit (Sigma Chemical Company).

Measurement of glycogen synthase and phosphorylase activities

Glycogen synthase and phosphorylase activities were determined according to the methods of François, Villanueva & Hers (1988). Glycogen synthase activity was established by measuring glucose incorporation into glycogen from UDP-[U-¹⁴C]glucose and glycogen phosphorylase activity was measured in the direction of glycogen synthesis by the incorporation of radioactively labelled [U-¹⁴C]glucose-1-phosphate into glycogen.

Measurement of α-amylase (1,4 α-D-glucan glucanohydrolase, EC 3.2.1.1)

Hydrolysis of *p*-nitrophenyl-α-D-maltoheptaoside was employed in attempts at α-amylase measurement, using a Sigma diagnostic kit (procedure No. 576). No α-amylase was detectable in extracts of *Coprinus* caps; purified commercial enzyme (human salivary α-amylase purchased from the Sigma Chemical Co.) was used in reconstruction experiments to demonstrate that none of the extraction and preparative procedures specifically affected this enzyme activity.

RESULTS AND DISCUSSION

In order reliably to correlate glycogen metabolism with development of different fruit bodies an objective time-course of development was needed. Hammad *et al.* (1993*a*) made a study of 35 different fruit bodies, taking gill samples every 1 to 2 h throughout the progress of meiosis and basidiospore formation to establish the exact time-course of readily observed cytological features. This has been used here as the basis for the developmental time-frame of glycogen metabo-

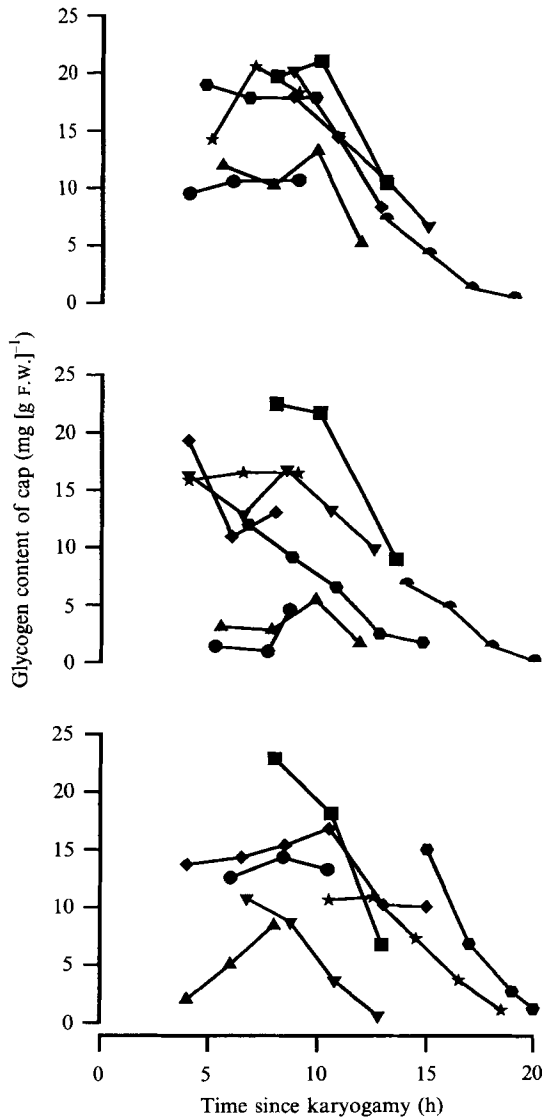


Fig. 1. Glycogen metabolism during meiosis and sporulation in 23 fruit bodies of the wild-type Meathop dikaryon of *Coprinus cinereus*. In each panel each symbol represents a different fruit body.

lism. With karyogamy used as the reference point of time zero, meiosis I started at 5 h, meiosis II at 6 h, sterigmata began to emerge after 7.5 h, basidiospore formation started at 9 h, basidiospore pigmentation at 11 h and basidiospore discharge at 18 h.

Glycogen content of the fruit body cap during meiosis and sporulation

Individual fruiting bodies were repeatedly sampled for glycogen assay by removing a few gills every 2 h until the fruit body matured; this manipulation did not affect the progress of fruit body maturation. The results from 23 fruit bodies sampled in this way (Fig. 1) show that glycogen content varied greatly between individual fruit bodies in an absolute sense (for example, some fruit bodies contained 20–23 mg glycogen g⁻¹ F.W. when sterigmata first appeared, others contained only 10% of that quantity). More importantly, the accumulation of glycogen in the caps was not

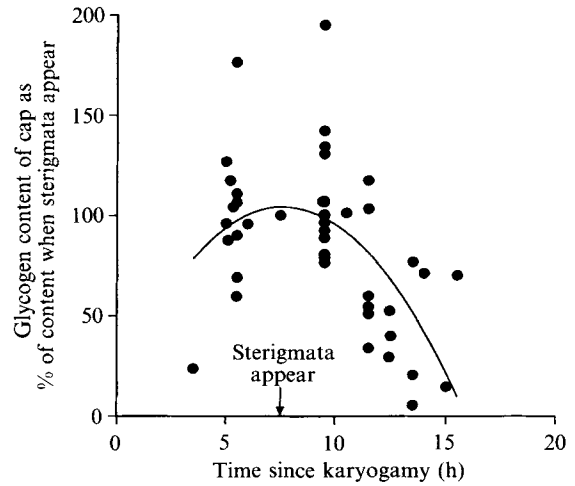


Fig. 2. Comparison of glycogen metabolism in 13 of the data sets of Fig. 1 which shared a measurement at the time that sterigmata appeared (7.5 h after karyogamy). To bring the observations to a common quantitative scale this (sterigmata appearance) point has been set at 100% and the other values in each set scaled accordingly. The curve shown has been fitted as a polynomial regression. It is very similar to curves published previously which claim a relationship between glycogen metabolism and the progress of meiosis and sporulation but as less than 40% of the variance can be accounted for by the relation it is considered to be spurious.

synchronized with a particular stage of meiosis. Nevertheless, 17 fruit bodies showed an increase in glycogen during meiosis and every fruit body showed a decrease in glycogen when the fruit body was sampled after sporulation. Although the data can be combined to suggest a relation between the progress of meiosis and glycogen accumulation and loss (Fig. 2), the coefficient of determination for the regression was only 0.39. Since 60% of the variance remained unaccounted for by this relation, it must be considered spurious. In many fruit bodies glycogen accumulation continued through meiosis to sterigmata appearance. This was the first indication that cap glycogen accumulation was not halted by the completion of meiosis. The stage at which glycogen content began to decline varied in different fruit bodies between 7 and 15 h after karyogamy. If glycogen accumulation and degradation were an integral part of meiosis, closer synchrony between the two processes might have been expected. However, in no fruit body was there an increase in glycogen accumulation after spore formation.

Glycogen content of fruit body caps during sporulation in vitro

Glycogen content of the cap rapidly decreased after explantation onto both plain buffered medium and buffered medium containing ammonium chloride (Fig. 3). On both media the glycogen content of the gills declined to about one third, 2 to 3 h after explantation; after 9 to 10 h glycogen content was minimal. Addition of ammonium ions to the explantation medium is known to inhibit sporulation (Chiu & Moore, 1988b, 1990a) but the pattern of glycogen accumulation in such tissues was very similar to that in tissues

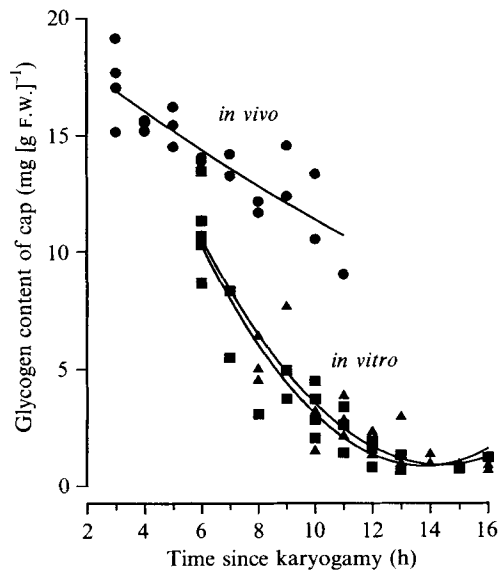


Fig. 3. A comparison of glycogen metabolism *in vivo* (circles; recording glycogen contents in successive fragments from the same fruit body) and *in vitro* (recording glycogen contents in gills excised 6 h after karyogamy and transplanted to plain buffer medium (squares) or ammonium medium (triangles)).

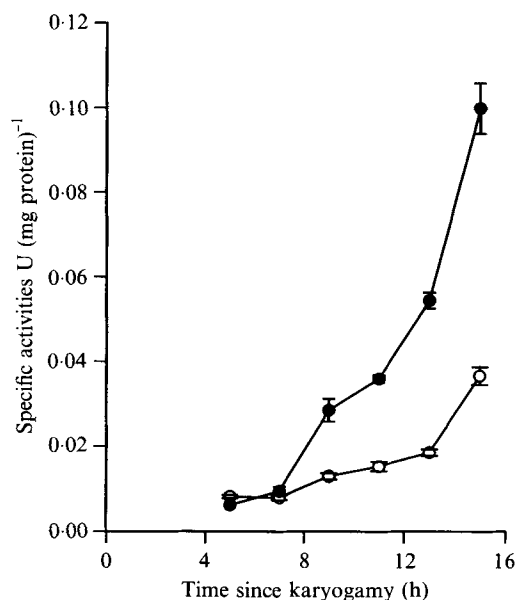


Fig. 4. Specific activity of glycogen synthase (open symbols) and glycogen phosphorylase (closed symbols) in caps of *C. cinereus* fruit bodies at various stages of development. Errors bars represent SEM ($n = 4$).

explanted to plain buffer medium (Fig. 3). Mature, blackened basidiospores were observed on gills explanted to plain buffered agar, but on ammonium-supplemented agar microscopic examination confirmed that most of the basidia had formed sterigmata but no basidiospores. These results indicate that although ammonium ions inhibit sporulation in *C. cinereus*, there is no consequential effect on the glycogen content of the gills. The fact that the glycogen stores of the gills underwent similar degradation on both plain and

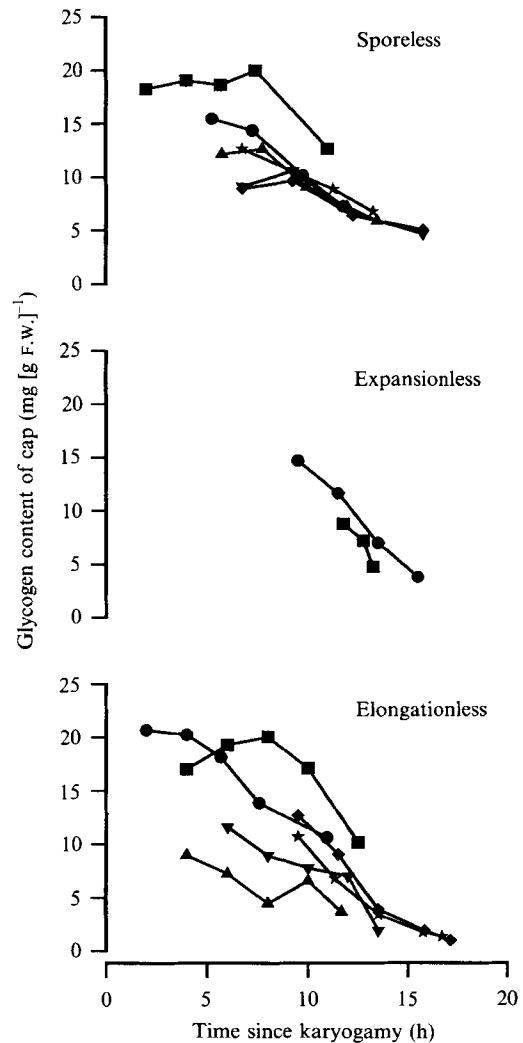
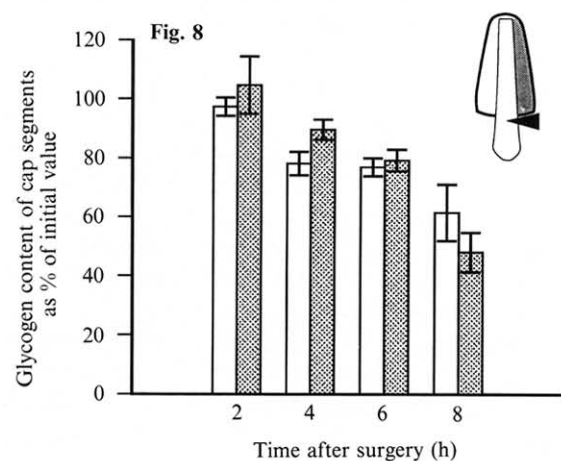
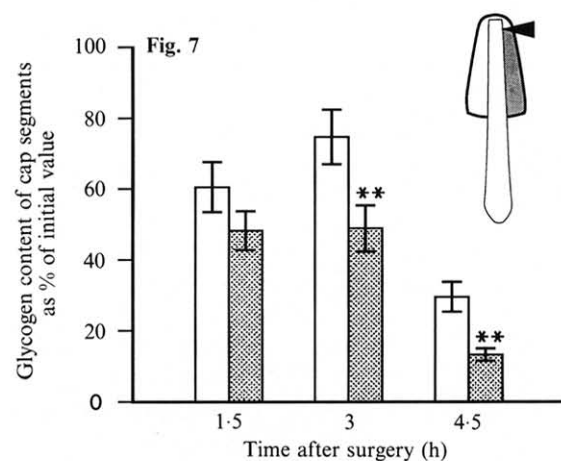
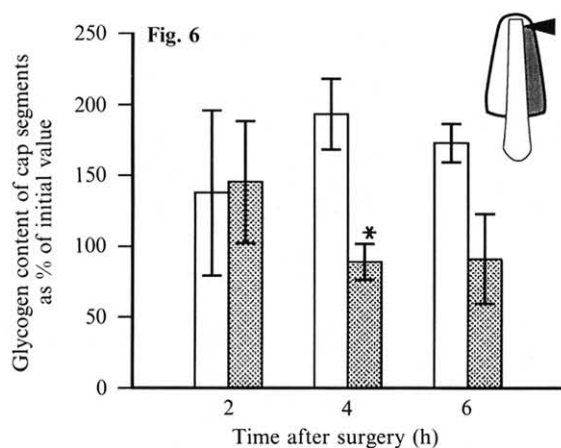


Fig. 5. Glycogen contents of fruit bodies of strains carrying the dominant developmental mutations sporeless, expansionless or elongationless. In each panel each symbol represents a different fruit body. Compare the patterns of glycogen content during fruit body development with the wild type shown in Fig. 1.

ammonium supplemented medium indicated that glycogen degradation and sporulation were not coupled together as closely as they are in yeast. 'Degradation' is stressed because the analytical method used specifically measured high molecular weight glycogen (the polysaccharide was alcohol-precipitated before being brought back into solution for reaction with the reagents). Therefore, what has been demonstrated is that ammonium did not interfere with the processes which reduced the concentration of precipitable, iodine-reacting polysaccharide. The high molecular weight glycogen might have been degraded into smaller molecules and ammonium may have affected utilization of these (unmeasured) fragments.

Measurement of enzymes involved in glycogen metabolism

Glycogen synthase and phosphorylase activities were measured during fruit body maturation (Fig. 4). Glycogen phosphorylase activity first increased when sterigmata ap-



Figs 6–8. Effects of surgery on the translocation of carbohydrate. Horizontal tangential cuts were made in fruit bodies with sterile scalpels and pieces of sterile aluminium foil inserted into the cuts to interrupt potential translocation routes. The cartoons in each panel show where the implants were made in each case. Glycogen contents were determined in slivers of cap material removed, at different times following surgery, from the uncut and cut segments of the fruit bodies. **Fig. 6** compares glycogen content of the uncut section (open bars) and the cut section (shaded bars) of young fruit bodies (4.5 h after karyogamy) in which the surgery was intended to interrupt any flow occurring through the cap flesh radially from the apex towards the cap margin. Error bars represent SEM ($n = 4$). **Fig. 7** shows a similar comparison for more mature fruit bodies (11.5 h after karyogamy at time of surgery); error bars represent SEM ($n = 6$). In **Fig. 8** the effect of stem incision is examined. Bars compare glycogen content in the cap segment immediately above the stem

peared (7.5 h after karyogamy); activity increased more than tenfold between then and basidiospore maturation. Glycogen synthase activity was measured in the same fruit bodies and the results showed that activity was low during sporulation but there was a 4-fold increase during basidiospore maturation, indicating that glycogen synthesis and degradation could occur simultaneously and that the ability to synthesize glycogen was maintained throughout the period when glycogen degradation was most rapid.

No α -amylase activity was detectable in cell-free extracts of *C. cinereus* fruit body caps. The sample extract did not inhibit activity of commercial pure α -amylase.

Glycogen contents of fruit bodies of strains carrying dominant developmental mutations

The general pattern of glycogen accumulation during meiosis and degradation some 6 to 9 h after karyogamy which was characteristic of the development of wild-type fruit bodies, was also seen in fruit bodies of strains genetically incapable of stem elongation, cap expansion or basidiospore formation (Fig. 5). There was an obvious rise and fall in the glycogen content of the non-sporulating strain NG 107 during meiosis and later cap development. The similarity of the patterns of glycogen metabolism in the wild-type strain and the non-sporulating strain provided a further indication that glycogen accumulation and degradation were not specifically associated with the sporulation process. Results with the other mutants also suggest that, at least for these particular alleles, there is no strict association between glycogen degradation and either cap expansion or stem elongation.

Measurement of nutrient exchange between cap and stem

A comparison of the variations in glycogen content with time between normally developing fruit bodies (the *in vivo* experiment) and tissue explanted to agar media (the *in vitro* experiments) (Fig. 3) shows that they differed markedly. The rate of glycogen degradation *in vitro* was much greater than the rate of degradation occurring in gills *in vivo*, that is, in gills left on the fruit body. About 11 h after karyogamy, only about 60% of the glycogen had been degraded *in vivo* (Fig. 3) whereas almost all of the glycogen was degraded *in vitro*. A possible explanation is that, *in vitro* the gills could not obtain translocated materials from other parts of the fruit body (as they might in the intact fruit body), so the net glycogen utilization was high. An experiment was carried out to test this hypothesis by surgically interrupting translocation to the gills.

Surgically treated fruit bodies

Surgical treatments were performed on both caps and stems to try to block potential translocation routes. Small pieces of

incision (shaded bar) with the content of the cap segment above the intact stem. Error bars represent SEM ($n = 5$). Single asterisks denote a significant difference (*t*-test) between cut and uncut segments at the 5% level, and double asterisks a significant difference at the 1% level.

aluminium foil were inserted into tangential incisions (see cartoons in Figs 6–8) in caps of fruit bodies 4.5 h after karyogamy. The fruit bodies were then kept at 26° in an illuminated incubator and glycogen content in the cap was measured every 2 h (Fig. 6). After 2 h the glycogen concentrations in the surgically treated and untreated segments were not significantly different ($P > 0.05$); after 4 h the surgically treated segment contained less glycogen than the untreated segment ($P < 0.05$); and 6 h after surgery there was, again, no significant difference ($P > 0.05$) between the two segments. When the same type of surgery was performed on fruit bodies in which basidiospores were already pigmented (about 11 h after karyogamy) (Fig. 7) there was no significant difference between the segments after 1.5 h but after 3 and 4.5 h further incubation the part of the cap beneath the inserted aluminium foil had a significantly lower glycogen content than the untreated part of the cap ($P < 0.01$).

In experiments with young fruit bodies, therefore, a transient reduction in glycogen content seemed to be compensated so that 6 h after surgery the glycogen content of the cap below the incision was not significantly different from the control sample. In contrast, in similar experiments with older fruit bodies the glycogen content below the incision was very significantly different from that of the control 4.5 h after surgery. In young fruit bodies the margins of primary gills are connected to the stem, tramal hyphae interweaving with the stem hyphae; as the fruit body matures these connections are broken (Rosin & Moore, 1985; Moore, 1987; Chiu & Moore, 1990b). The results of our surgical experiments imply that in the younger fruit body cap, lateral translocation of material was more likely to occur than in an old fruit body cap; materials could be translocated between the gills or between stem and gill in the young cap, so bypassing the incision and aluminium foil insert. Detached from the stem, the older cap is limited to radial translocation routes (i.e. from apex to margin) through the cap flesh and these were successfully blocked by the insert. In other words, net translocation of glycogen from stem to cap is indicated, continuing well beyond formation and pigmentation of basidiospores. Even the mature cap (which can contain up to 95% of the total glycogen of the fruit body) remains dependent on translocation of carbohydrate from the stem to maintain those reserves.

Stems were also surgically treated with incisions and horizontal insertions of aluminium foil to compare the cap glycogen content about the side of the stem in which the incision was made with that above the untreated side (Fig. 8). No significant difference was detected between glycogen contents of the two segments of the cap. This suggests that nutrients were translocated around the surgical barrier. Hammad, Watling & More (1993b) have shown that the stem in *C. cinereus* has a large population of narrow hyphae which interweave and interconnect between the inflated hyphae which make up the bulk of the volume of the stem. These surgical experiments suggest that the network of interconnections in the stem is able to translocate nutrients around inserted barriers.

The above experiments dealt with stem to cap translocation but some of the data could be taken to imply translocation of

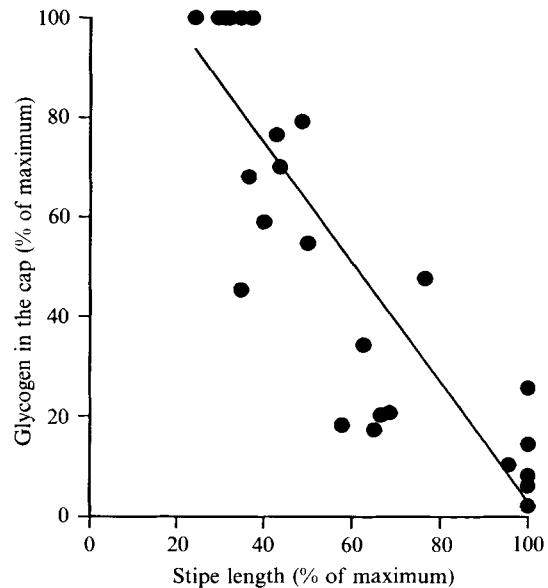


Fig. 9. The apparent correlation between glycogen content and stem length in *C. cinereus*. A fairly high negative correlation ($r^2 = 0.76$) implies a relationship between stem length and the amount of glycogen in the cap but the fact that stem elongation proceeds in decapitated fruit bodies suggests that the relation is coincidental rather than causal.

glycogen in the reverse direction. Hammad *et al.* (1993a) have shown that the phase of rapid stem elongation seemed to be correlated with the ending of meiosis. Indeed, there is a better correlation between glycogen content of the cap and stem elongation (Fig. 9; coefficient of determination, $r^2 = 0.76$) than between glycogen content and any other feature. This could suggest the hypothesis that cap glycogen might be degraded and transferred back to the stem, for use in stem elongation. Gooday (1974) found that stem elongation in *C. cinereus* had no requirement for connection either with the cap or the parental mycelium. Hammad *et al.* (1993a) demonstrated another aspect of this cap/stem interplay by showing that whilst the stem may not be dependent on the presence of the cap, its elongation certainly benefits considerably from the presence of the cap: intact fruit bodies elongated about 25% more than decapitated ones (amounting to 2 to 3 cm greater length).

Another attempt to determine whether nutrients were translocated, in either direction, between caps and stems involved prelabelling fruit bodies with ^{14}C and then performing graft interchanges (labelled cap to unlabelled stem and vice versa, as well as labelled cap to artificial stem and artificial cap to labelled stem). Results showed that even though the cap contained more ^{14}C than the stem ($6 \pm 1 \times 10^5$ cpm g^{-1} f.w. compared with $4 \pm 0.3 \times 10^5$ cpm g^{-1} f.w. [all values are means \pm SD of 5 replicates]), the real cap received more ^{14}C from the labelled stem (1082 ± 616 cpm g^{-1} f.w.) than the real stem did from the labelled cap (291 ± 149 cpm g^{-1} f.w.). Similarly, even the artificial cap received more ^{14}C (557 ± 174 cpm g^{-1} f.w.) than the artificial stem (315 ± 93 cpm g^{-1} f.w.). These results demonstrated that any nutrient translocation which might

occur under such conditions was primarily in the direction from stem to cap. However, the quantities transferred were extremely small (0.05 to 0.3% of the total label). On the other hand, if the differences in glycogen contents between surgical treatments and controls in Fig. 7 can be taken as a measure of translocation flow they suggest that 35% of the glycogen is supplied this way. One difference (among many) between the situations, of course, is that the translocation of ^{14}C had to occur extracellularly, across a graft junction. The paucity of this flow suggests that if significant quantities of nutrients are moved between different parts of the fruit body the translocation stream must be within the hyphae.

Clearly, as fruit bodies develop to maturity cap glycogen shows a distinct synthesis-degradation cycle in *C. cinereus*, just as it does during mycelial development (Jirjis & Moore, 1976). The accumulation of glycogen, obviously requiring rapid synthesis and large-scale translocation, and its subsequent fast degradation gives the impression that this glycogen synthesis-degradation cycle serves a specific function during fruit body maturation like the sporulation-dependent degradation of glycogen observed in yeast (Kane & Roth, 1974). In general, however, the present work has demonstrated that the glycogen accumulated in the cap was required for all the processes of later cap development, i.e. basidiospore formation, cap expansion, stem elongation, etc., but was not specifically or exclusively utilized for any one of these processes. It is also clear that there is an organized intracellular nutrient translocation circuit from mycelium to stem to cap. However, there is reason to believe that the stem receives some nutrients from the cap and that, while the major supply route to the gills runs radially from the cap apex through the cap flesh, in young fruit bodies they can be serviced by translocation of nutrients through the connections which exist between the stem and the 'edges' of primary gills.

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