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# ELECTROPHORETIC STUDIES OF CARPOPHORE DEVELOPMENT IN THE BASIDIOMYCETE *COPRINUS CINEREUS*

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## SUMMARY

Acrylamide gel electrophoresis was used to resolve buffer-soluble proteins and enzymes extracted from tissues of *Coprinus cinereus*. The electrophoretograms of proteins of two related but compatible monokaryons differed from one another, and both differed from the electrophoretogram of the dikaryon made by crossing the monokaryons together. The electrophoretogram of the youngest carpophore primordia was markedly different from that of the dikaryon on which they were produced, and the protein spectrum of both cap and stipe altered as the tissues matured. The protein electrophoretic pattern observed in the cap was always different from that of the stipe on which the cap was borne. However, when carpophores of quite different dikaryons were compared it was found that differences between the strains were so considerable that it was not possible to identify features common to a particular tissue or structure. Variation was also found in respect of isozyme patterns even though only two quite closely related monokaryons were compared.

In the case of two enzyme activities (malate dehydrogenase and NADPH-dehydrogenase) the monokaryons exhibited different numbers of isozymes, and in a further three (alcohol dehydrogenase, NADH-dehydrogenase and esterase) there were clear differences in the way in which the isozyme pattern in each monokaryon related to the period of growth. The dikaryon was not associated with any predictable change in the isozyme pattern, but in all cases the dikaryon was in some way different from the parental monokaryons. Further differences were observed in carpophore tissues: in all cases the carpophore differed from its parental dikaryon. In most cases this difference was expressed as a distinctive developmental pattern of isozymes which were also detectable in mycelium; and in this the cap was often quite different from the stipe. However, for alcohol dehydrogenase and esterase, extracts of carpophore tissues possessed isozymes not detectable in extracts of mycelia.

## INTRODUCTION

Developmental studies with eukaryotic microbes have contributed greatly to our knowledge of the ways in which cellular functions are controlled and integrated in morphogenesis. Although many such micro-organisms have been used it is a surprising fact that the agaric fungi have so far been used very little for this type of work. Indeed, basidiomycete fungi do not figure at all in a recent review volume dealing with microbial development (O'Day and Horgen, 1977), and even in the most recent compilation of works on development in filamentous fungi (Smith and Berry, 1978) agaric morphogenesis is almost completely ignored, even though agarics are potentially ideal organisms for this sort of research.

During the past few years biochemical analyses of *C. cinereus* have revealed a number of metabolic activities which change drastically during morphogenesis. Interest at present is concentrated on enzyme sequences involved in nitrogen

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metabolism (Ewaze, Moore and Stewart, 1978). This interest arose from the discovery that the NADP-linked glutamate dehydrogenase of *C. cinereus* is found at high specific activity in mature carpophore caps, but is barely detectable in the tissues of the stipes which support those caps (Stewart and Moore, 1974). This enzyme activity is barely detectable in the very earliest primordial stages of the carpophore cap and undergoes a 20- to 50-fold increase in specific activity during development. It is thought to represent a specific development-related derepression with its regulatory origin at the genetic level. However, irrespective of the nature of the regulatory event, comparison of cap and stipe shows that contrasting regulatory patterns operate in these two adjacent tissues. Indeed, cytochemical examination reveals that adjacent cells may exhibit these same regulatory contrasts (Elhiti, Butler and Moore, 1979).

It is clearly important to known the extent of such metabolic peculiarities of different parts of the carpophore. Most of the data have so far been obtained by enzymological analyses, but a broader and slightly different view can be obtained by use of electrophoresis. In the study reported here electrophoretic analysis has been used to survey the soluble proteins of mycelium and carpophore to establish the extent of variation among different strains and tissues during development. Another aspect which is considered is the isozyme pattern of a number of enzyme activities. Although isozyme patterns have been studied in relation to dikaryon formation in two basidiomycetes (Wang and Raper, 1970; Ross, Martini and Thoman, 1973) and changes in isozyme pattern have been followed through morphogenesis of the asexual sclerotia of the ascomycete *Sclerotinia* (Wong and Willetts, 1974) no attempt has yet been made to investigate what changes might occur in isozyme pattern during development of the basidiomycete carpophore. In the work reported here we have investigated seven enzyme activities with respect to changing isozyme pattern during growth of two monokaryotic mycelia, during vegetative growth of their joint dikaryon, and in the caps and stipes of carpophores developing on that dikaryon.

## MATERIALS AND METHODS

### *Organism*

The organism used was *Coprinus cinereus* (Schaeff. *ex* Fr.) S. F. Gray *sensu* Konr. It has previously been called *C. lagopus* (e.g. Stewart and Moore, 1974) and in Japan the same species is used under the name *C. macrorhizus* (e.g. Ishikawa and Uno, 1977). However, all of these strains are cross-fertile (Moore, Elhiti and Butler, 1979) and are more correctly called *C. cinereus* (Pinto-Lopes and Almeida, 1970). The strains used here were the monokaryons H1 (mating type $A_5B_5$), BC9/6,6 ($A_6B_6$) and ZBw601/40,40 ($A_{40}B_{40}$) together with the dikaryons BC9/6,6+H1, BC9/6,6+ZBw601/40,40 (= dikaryon RA1 of Moore and Jirjis, 1976) and the dikaryon 601 which was isolated in Czechoslovakia by J. Necasek and from which monokaryon ZBw601/40,40 was derived (Moore and Stewart, 1972). Vegetative cultures and carpophores were grown as described by Jirjis and Moore (1979).

### *Electrophoresis*

Cell-free extracts were prepared, desalted, and subjected to electrophoresis using the techniques described by Jirjis and Moore (1979) except that in some experiments described below the acrylamide concentration was varied. For general

proteins gels were stained with Gurr's Naphthalene Blue Black 10B. Enzyme activity in the gels was detected using assay mixtures modified from those reported by Cole, Blondin and Temple (1968) and Shaw and Prasad (1970). Protein contents of the extracts were measured bythe method of Lowry *et al.* (1951) and the specific activities of individual enzyme bands calculated with the aid of densitometric records as described earlier (Jirjis and Moore, 1979). It is important to emphasize that throughout the preparative stages (extraction, desalting and electrophoresis) the temperature of the material did not exceed 4 °C. Glutamate dehydrogenase activity was assayed in each of the different types of material described and both GDH enzymes were always found to migrate as single bands (Jirjis and Moore, 1979).

## Results

### *Proteins of vegetative mycelium*

The monokaryons H1 and BC9/6,6 and their joint dikaryon, BC9/6,6 + H1 were grown on YMG liquid medium and at regular intervals during incubation samples were removed, extracted, and the proteins separated on 7·5 % polyacrylamide gels (Fig. 1). Although some difference was evident between the two

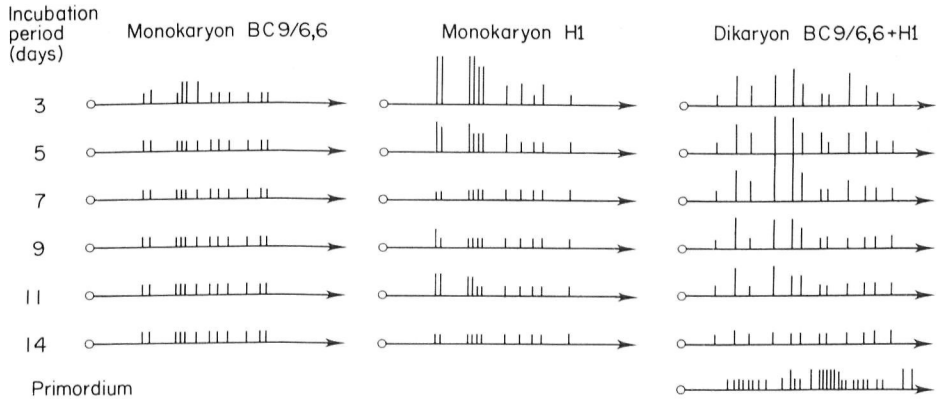


Fig. 1. Electrophoretograms of buffer-soluble proteins extracted from mycelia of *Coprinus cinereus*. The relative positions and staining intensities of protein bands detected by densitometer are indicated by the position and height of the vertical bars; staining intensity measurements being corrected to a common scale. The origin is shown as an open circle and the direction of migration (towards the anode) by the arrowhead. The results depicted show that the two monokaryons differed from each other and from their joint dikaryon, and that the tissue of a stage 1 carpophore primordium (2 to 6 mm tall, prekaryogamy) differed from its parental mycelium.

monokaryons, especially in respect of the intensity of staining, their electrophoretograms were very similar and both showed a general decline in staining intensity over the period of incubation. However, the pattern obtained from the dikaryon differed radically from those of the monokaryons used in its formation. Profound differences were evident in the distribution of the bands, and although a general decline in intensity of staining was evident over the second week of incubation, the dikaryon was exceptional in having three bands which showed a steady increase in stain intensity during the first week. Another striking developmental aspect was that the electrophoretogram of extracts of stage 1 primordia differed greatly from that of the parental dikaryotic mycelium.

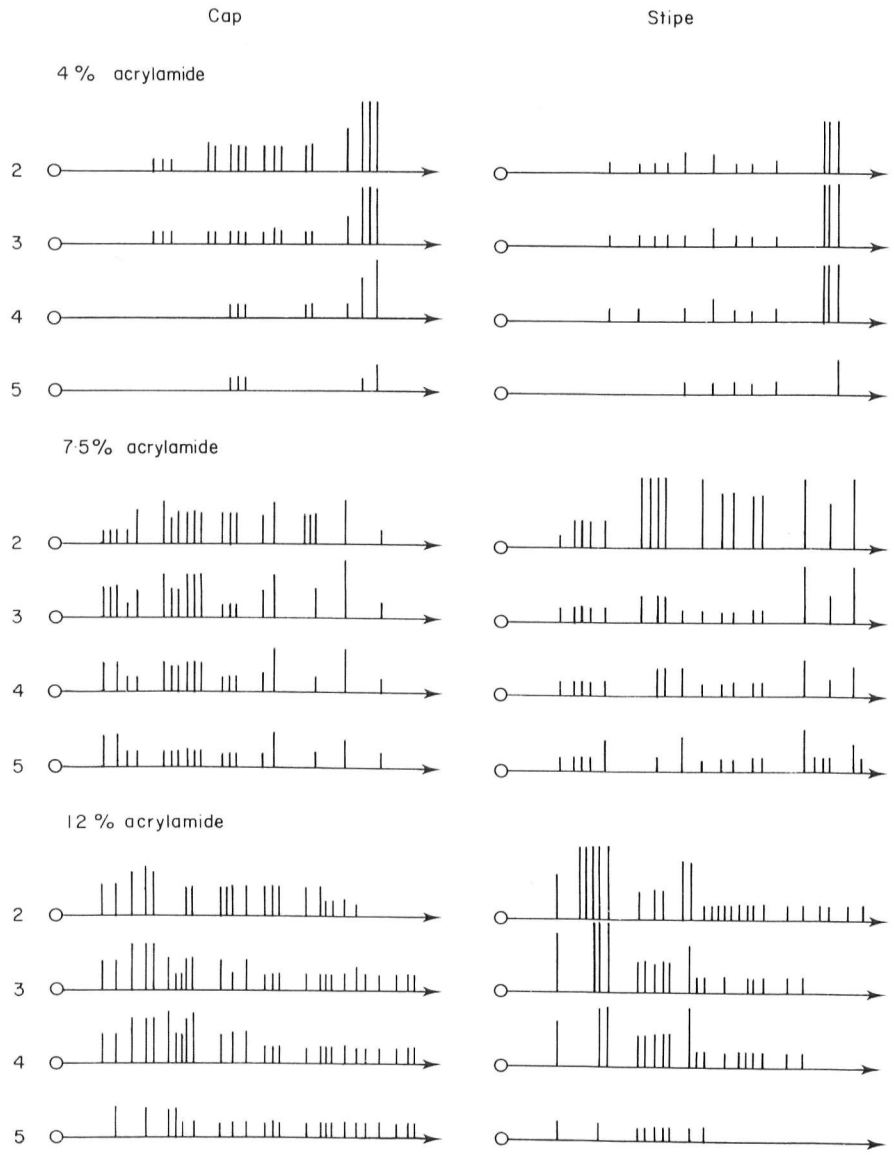


Fig. 2. Use of different acrylamide concentrations to separate buffer-soluble proteins extracted from carpophore tissues of *Coprinus cinereus*. Four different developmental stages of the carpophore are represented: stage 2 (6 to 9 mm tall, in which karyogamy and meiosis occur), stage 3 (over 10 mm tall, post-meiotic, partial veil free), stage 4 (20 to 40 mm tall, the stage in which spores become pigmented), and stage 5 (mature carpophores, up to 100 mm tall, discharging spores). All the specimens were produced on the dikaryon BC9/6,6+ZBw601/40,40. Diagrammatic conventions as for Fig. 1.

*Proteins of the carpophore*

Carpophores produced by three different dikaryons were examined. In each case different developmental stages were studied, development being categorized into the stages described by Stewart and Moore (1974). In this system the youngest recognizable primordium is assigned to stage 1 and is typically 2 to 9 mm tall,

whereas the mature spore-discharging fruit body, which may be more than 100 mm tall, is described as stage 5. In all except stage 1 primordia the carpophore caps were surgically removed from their stipes and the two organs analyzed separately. Material obtained from the dikaryon BC9/6,6+ZBw601/40,40 was electrophoresed on three different gel concentrations (Fig. 2) and all three allow interesting comparisons to be made. Generally, a large population of (low molecular weight) proteins was observed in 12% polyacrylamide gels and a small population of (high molecular weight) proteins was recovered in 4% gels. Interestingly, the number of protein bands visible in 4% gels was reduced as increasingly mature tissue was examined, while, at least in the carpophore cap, the number of bands in 12% gels increased with increasing maturity of the tissue. The 7·5% gels were considered to give a sufficiently representative picture for this concentration of acrylamide to be used to compare the carpophores of other dikaryons (Fig. 3). In all cases the electrophoretic pattern of the cap differed radically from that of its stipe, and in both organs the pattern altered as the tissues matured. However, it is significant that the patterns observed in the three different dikaryons were more diverse than they were similar.

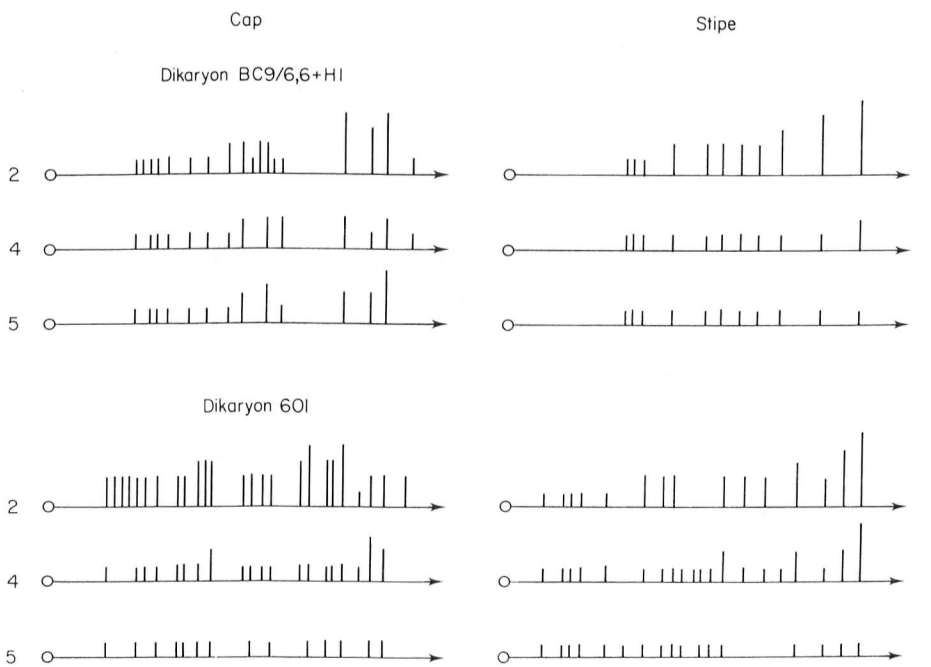


Fig. 3. Comparison of electrophoretograms of carpophore tissues produced on two different dikaryons.

*Enzymes of mycelium and carpophore*

*NAD-linked alcohol dehydrogenase.* Four ADH isozymes were detected in mycelial extracts. The relative positions of the isozymes were defined as the ratio between the migration of the enzyme band and migration of the bromophenol blue tracking dye. These $R_P$ values for the ADH isozymes from mycelial extracts were ADH-1 0·15, ADH-2 0·23, ADH-3 0·28 and ADH-4 0·35. All four isozymes were

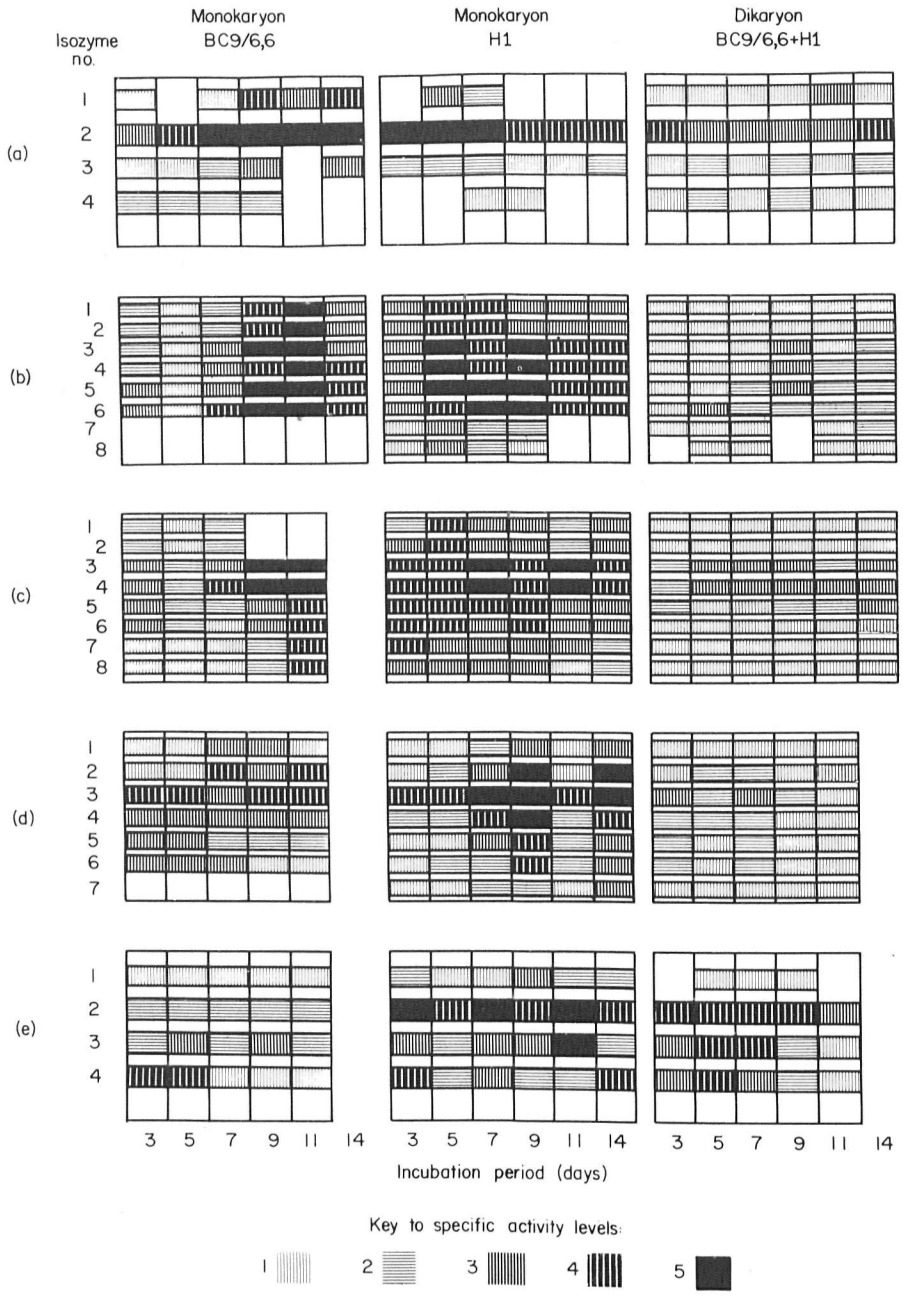


Fig. 4. Isozyme spectra of mycelia of *Coprinus cinereus*. Mycelia were harvested after 3 to 14 days incubation in liquid medium and buffer extracts subjected to electrophoresis. The gels were then stained to show activity of (a) alcohol dehydrogenase, (b) malate dehydrogenase, (c) NADH-dehydrogenase, (d) NADPH-dehydrogenase, and (e) glucose 6-phosphate dehydrogenase. The activity of each isozyme was measured densitometrically and corrected to a common notional initial protein loading. The specific activity of each band is indicated in the diagram by assignment to the five different shading categories: activity level 1 = change of up to 0·25 A in optical density per mg protein during 20 min incubation; level 2 = change of 0·25 to 0·5 A; level 3 = change of 0·5 to 1·0 A; level 4 = change of 1·0 to 2·0 A; level 5 = change of greater than 2 A. The diagram does not attempt to depict the relative positions of the isozymes; this information is given in numerical terms in the text.

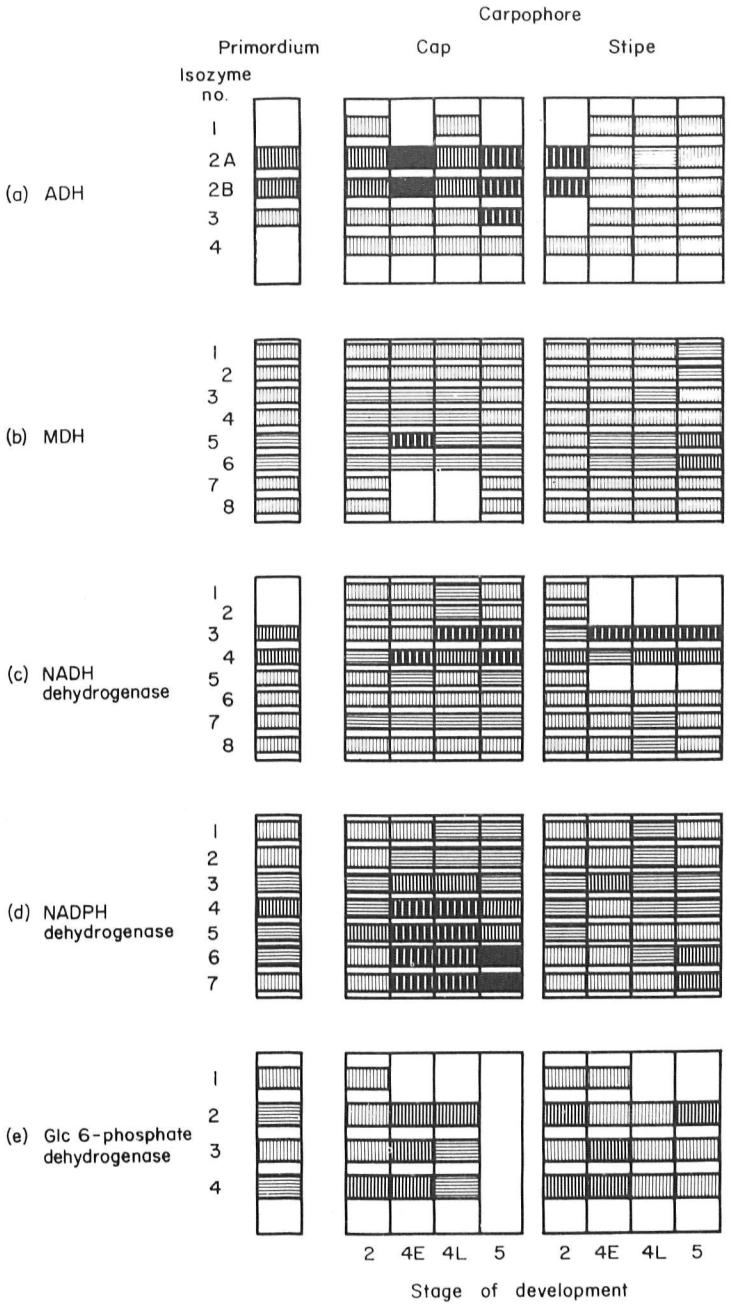


Fig. 5. Isozyme spectra of carpophore tissues of *Coprinus cinereus*. The same enzyme activities are shown here as appear in Fig. 4, and specific activities of individual isozymes are indicated in the same way. Carpophore primordia of stage 1 (2 to 6 mm tall, prekaryogamy) were extracted whole, without dissection. The other carpophore stages were dissected into caps and stipes before extraction. Four developmental stages were examined; stage 2 (6 to 9 mm tall, karyogamy and meiosis), stage 4 E (22 to 25 mm tall, spore pigmentation just beginning), stage 4 L (up to 40 mm tall, with spores fully pigmented), and stage 5 (mature fruiting body, up to 100 mm tall, discharging spores). The carpophores were grown on the dikaryon BC9/6,6+H1.

always detected in dikaryon extracts, but the monokaryons differed from one another in their isozyme spectrum during the 14-day incubation period [Fig. 4(a)], H1 in particular frequently lacked detectable activity of isozymes ADH-1 and ADH-4. Despite the differences between the monokaryons the generalization can be made that ADH-2 and ADH-3 gradually increased in activity over the first 7 to 9 days incubation; this was clearly different from the dikaryon in which ADH-2 was at its highest specific activity when first sampled after 3 days incubation. Another point of difference between the monokaryons and their dikaryon is that the specific activity of ADH-2 was much higher in the monokaryons than in the dikaryon. Strain BC9/6,6 showed the highest level with a specific activity (of ADH-2) of 0·47 optical density units $min^{-1}$ mg $protein^{-1}$. In the same units, the maximum specific activity of ADH-2 in H1 was 0·18 and in the dikaryon 0·06. This peculiarity applied only to ADH-2. The other isozymes, though varying in activity with time and between different mycelia, generally showed activity maxima at similar levels in all three mycelia. The ADH-2 isozyme was also unusual in extracts of carpophores. Although isozyme bands with identical $R_P$ values to the ADH-1, ADH-3 and ADH-4 mycelial isozymes were detected, two separate bands of $R_P$ value 0·23 and 0·25 appeared in all carpophore extracts and were always the most active enzyme bands in the gel, though generally of equal activity when compared with one another. Although we have no evidence for any relationship between these two isozymes, in the drawings in Figure 5(a) we categorize them for convenience as ADH-2A ($R_P$ value 0·23) and ADH-2B ($R_P$ value 0·25). It is interesting to note that ADH-2A and ADH-2B reach their highest specific activities in the stipe at primordium stage 2 whereas in the cap the highest activities are attained much later, at stage 4.

*NAD-linked malate dehydrogenase.* The number of isozymes of MDH which were detected differed between the two monokaryons studied. Extracts of the mycelium of strain BC9/6,6 revealed six isozymes in all samples. Six enzyme bands with the same $R_P$ values were also found in extracts of strain H1 mycelium, but in addition a further two isozymes were apparent in samples taken during the first 9 days of incubation, although they faded subsequently [Fig. 4(b)]. These eight isozymes were also found in the dikaryon constructed from the monokaryons. The MDH isozymes were found to have $R_P$ values of 0·12 (MDH-1), 0·14 (MDH-2), 0·22 (MDH-3) 0·24 (MDH-4), 0·26 (MDH-5), 0·33 (MDH-6), 0·38 (MDH-7) and 0·41 (MDH-8). The specific activities of the MDH isozymes varied during mycelial growth. There was no clear pattern to the variation, but the monokaryons differed from one another and the dikaryon pattern was more akin to that of BC9/6,6 than to H1. All eight isozymes were found in carpophore extracts [Fig. 5(b)] although MDH-7 and MDH-8 were absent from extracts of stage 4 caps despite their presence in the stipes of the same carpophores. The pattern of specific activities differed between cap and stipe. In the stipe all of the isozymes gradually increased in activity as the tissue matured. MDH-6 followed the same pattern in the cap, but MDH-1 to MDH-5 increased in activity up to early stage 4 and declined in activity as the material further matured.

*Reduced nicotinamide adenine dinucleotide dehydrogenase.* Eight isozymes were detected with $R_P$ values of 0·23, 0·26, 0·32, 0·33, 0·38, 0·40, 0·44 and 0·47. All showed moderate to high levels of activity in samples of H1 mycelium taken during the first week of growth, and a general decline in activity subsequently. The other

monokaryon, BC9/6,6 showed the reverse pattern for most of the detected isozymes. In this strain isozymes 3 to 8 showed a considerably greater activity in the second week than in the first. However, isozymes 1 and 2 though present intially, were absent from samples taken during the second week. In the dikaryon all eight isozymes were present in all samples examined and the pattern of their activities was different in detail from both parental monokaryons [Fig. 4(c)]. A feature of the carpophore samples was the complete absence of three of the isozymes (nos 1, 2 and 5) from the later stages of stipe development. All eight isozymes were present in the primordial stipe and were represented in all stages of cap development [Fig. 5(c)]. A peculiarity of staining for NADH-dehydrogenase activity was that in all samples an undefined broad area of staining appeared at the anodal end of the gel. This remained foggy and ill-defined in all mycelial samples but became resolved into bands in caps and stipes of later stages of development. Although still poorly resolved, two bands at $R_P$ values of 0·59 and 0·61, together with others less well defined and remaining as shoulders on the major densitometer peaks, were evident in extracts of caps and stipes of stages 4 and 5.

*Reduced nicotinamide adenine dinucleotide phosphate dehydrogenase.* Six isozymes of this enzyme activity were found in extracts of strain BC9/6,6: these had $R_P$ values of 0·26, 0·29, 0·37, 0·42, 0·46 and 0·51. A seventh isozyme ($R_P$ 0·54) was detected in extracts of the other monokaryon, H1; it was also found in the dikaryon and in all carpophore samples examined. In extracts of mycelium isozyme 3 ($R_P$ 0·37) generally exhibited the highest specific activity [Fig. 4(d)]. This was also generally true for the carpophore stipe, but in the cap isozymes 4 to 7 showed much the highest specific activities, particularly at later stages of development [Fig. 5(d)]. Although these same isozymes increased in activity during development of the stipe also, such increase was on a much lesser scale than was seen in the developing cap and the quite large difference between cap and stipe in the relative increase in activity of these four faster migrating isozymes is the most striking feature of the observations made on this enzyme activity.

*Glucose 6-phosphate dehydrogenase.* Four isozymes with this enzyme activity were detected in samples of all tissues examined, although the appearance of isozyme 1 varied with incubation period in the dikaryon mycelium [Fig. 4(e)] and with the stage of development in carpophore samples [Fig. 5(e)]. The isozymes had $R_P$ values of 0·25, 0·30, 0·37 and 0·41. In mycelia the specific activities of individual isozymes varied but with no clear pattern. In the carpophore there was a distinct tendency for isozyme 2 to become dominant. This was evident in both cap and stipe but reached its greatest expression in the latter. In stipes taken from nearly mature carpophores isozyme 2 possessed in excess of 80% of the total activity detectable. Interestingly, no activity at all was detectable in extracts of caps removed from these same stipes.

*Esterase.* A total of 14 bands showed esterase activity in extracts of mycelia. Relative mobility in the gel was estimated using isozyme 5 as reference and the values obtained for isozymes in mycelial extracts were 0·62, 0·69, 0·81, 0·9, 1·0, 1·14, 1·2, 1·38, 1·47, 1·57, 1·71, 1·9, 2·0 and 2·14. The two monokaryons differed greatly in the way in which the specific activities of the isozymes varied with incubation period, and the dikaryon showed elements of the behaviour of both its parents [Fig. 6(a)]. Extracts of the carpophore also possessed 14 isozymes of

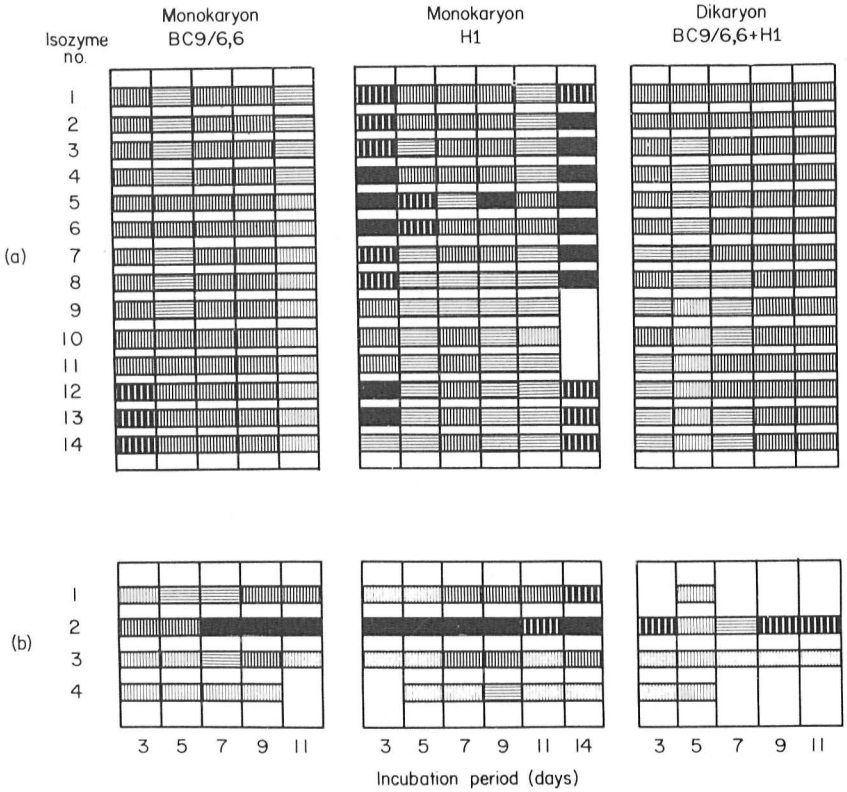


Fig. 6. Esterase (a) and acid phosphatase (b) isozymes of *Coprinus cinereus* mycelia. Specific activity levels are indicated as detailed in the legend to Fig. 4.

esterase activity. The first five were indistinguishable from the corresponding isozymes of mycelial extracts but the other nine were very different. The relative migrations (again referred to isozyme 5) were 0·62, 0·69, 0·81, 0·9, 1·0, 1·02, 1·05, 1·1, 1·2, 1·28, 1·38, 1·57, 1·95 and 2·1. All isozymes were detected in all samples of caps and stipes examined [Fig. 7(a)] and though the specific activities varied with developmental stage there was no clear trend to the variation.

*Acid phosphatase.* Acid phosphatase activity appeared in most samples in four isozyme bands. Relative mobilities were calculated by reference to the position of isozyme 4 and it appeared that isozymes of identical mobilities were to be found in extracts of all tissues examined. They had relative mobilities of 0·48, 0·57, 0·64 and 1. A peculiar feature of the mycelial samples was that although in both monokaryons isozyme 1 had a moderate specific activity which generally increased during incubation, this isozyme was detected at only one stage during the growth of the dikaryon. However, it must be noted that the dikaryon showed generally much reduced specific activities for this enzyme activity [Fig. 6(b)]. Caps and stipes were broadly similar in their patterns of specific activities for the acid phosphatase isozymes though maximum activities in the cap were reached rather earlier than in the stipe and then declined much more abruptly [Fig. 7(b)].

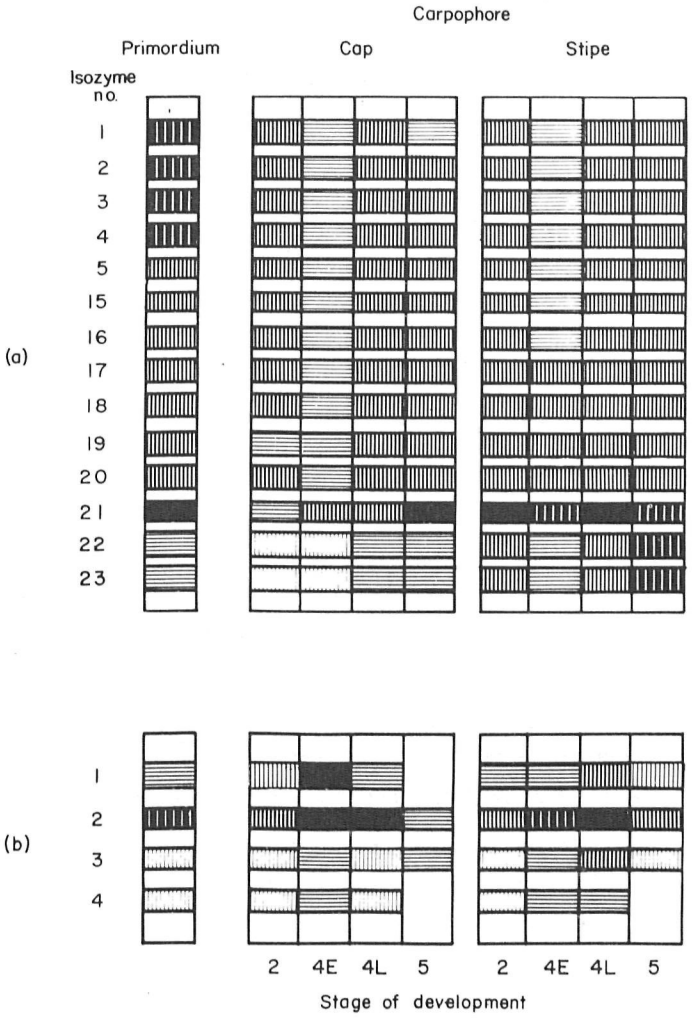


Fig. 7. Esterase (a) and acid phosphatase (b) isozymes of *Coprinus cinereus* carpophore tissues. Diagrammatic conventions are the same as used in other figures. Note that nine of the esterase isozymes of the carpophore differed from those found in mycelial extracts (see text), hence the difference in the numbering scheme. The carpophores were grown on the dikaryon BC9/6,6+H1.

## Discussion

As noted by Ross *et al.* (1973) a technique which depends on extraction of only buffer-soluble proteins makes a very particular selection from the total population of proteins of the material subjected to analysis. The problem is heightened when (as here) no attempt is made to concentrate the extracts and by the very general nature of the separation and staining procedures. Because of these limitations detailed interpretation of data such as we present can be misleading. Nevertheless some very important generalizations can be made.

It is clear that the electrophoretogram of the dikaryon was greatly different from that of either of the parental monokaryons (Fig. 1), suggesting that formation of the dikaryon involves fundamental changes on a large scale to cell structure

and metabolism. Similar observations have already been made in *Schizophyllum* (Wang and Raper, 1969) and a bipolar *Coprinus* species (Ross *et al.*, 1973). However, from our observations we are unable to draw any conclusions about the involvement of the incompatibility factors. A similarly considerable difference in electrophoretic pattern was apparent between extracts of the dikaryon and of the carpophore primordium to which it gave rise. Subsequently, as the carpophore cap and stipe differentiated and matured, it was observed that electrophoretograms of cap and stipe were different and that both altered as development proceeded. This was true for the carpophores of all three dikaryons studied, and has also been observed during development of the paddy-straw mushroom *Volvariella volvacea* (Chang and Chan, 1973). Such observations clearly illustrate that sweeping changes in the nature and content of soluble proteins of the tissue accompany each major morphogenetic change between monokaryon, dikaryon, primordium and carpophore cap or stipe grades of development of the mushroom. Even though particular enzyme changes may be identified and thought to characterize specific developmental steps (Ewaze *et al.*, 1978), the overall scope of change is obviously very large indeed. Ross *et al.* (1973) claimed that dikaryons of their bipolar *Coprinus* showed an electrophoretic pattern characteristic of the dikaryon grade of organization. In our view such a generalization is dangerous unless clearly qualified by recognition of the considerable polymorphism which can be detected. The dikaryon pattern certainly differs from that of the monokaryotic parents, so it must be the case that formation of the dikaryon involves regulatory changes of the sort discussed by Ross *et al.* (1973). However, using the same organism that we have used (though under the name *C. lagopus*) Smythe and Anderson (1971) have shown in a comparison of only 12 monokaryons that 29% of electrophoretically detected protein bands were of variable occurrence; no consistent relation to mating type was observed and large differences were seen even between mycelia derived from spores belonging to the same tetrad. Such widespread polymorphism readily accounts for the differences between the three dikaryons illustrated in Fig. 3 above; but the existence of protein polymorphism on this scale makes it impossible to categorize an electrophoretic pattern as being universally characteristic of a particular grade of organization or stage of development.

As well as general proteins we have examined the isozyme spectrum for seven enzyme activities in this study. Isozyme data have been used in the past to make very specific statements about the metabolic status of fungal mycelium during morphogenesis (Wong and Willetts, 1974). We believe this approach to be unwise unless the way in which the isozymes arise is known. Multiple forms of a particular enzyme activity can result from a number of very different situations, and Scandalios (1974) has stressed the need for complementary genetic information about the nature of the isozymes if they are to be used fully in studies of differentiation. In no case do we have information about the nature of the isozymes detected during this investigation. We will therefore restrict our conclusions to generalizations.

It is clear that genetic polymorphism is no less frequent in *Coprinus* than in other organisms. Although only two, genetically related, monokaryons were compared in the case of two enzyme activities the monokaryons exhibited different numbers of isozymes (malate dehydrogenase and NADPH-dehydrogenase) and in a further three (alcohol dehydrogenase, NADH-dehydrogenase and esterase) there were clear differences in the way in which the isozyme pattern in each monokaryon related to the period of growth. The dikaryon grade of organization was not

accompanied by any predictable change in isozyme pattern, but in all cases the dikaryon was in some way different from the parental monokaryons thus emphasizing the distinction between the two types of mycelial growth. The most dramatic differences were observed in carpophore tissues. In all cases the carpophore differed from its parental dikaryon. In most cases this difference was expressed as a distinctive isozyme pattern in the carpophore, and in this the cap was often quite different from the stipe. For two enzyme activities (alcohol dehydrogenase and esterase) extracts of carpophore tissues possessed isozymes not detectable in mycelium. Our general conclusion from these observations is that *Coprinus cinereus* displays sophistication in the distribution and regulation of its enzyme activities to a degree often associated only with more exalted 'higher' eukaryotes.

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