Proportionality in the pattern of differentiation of the cellular slime mould
*Dictyostelium discoideum* and the time of its determination

By PAUL A. FARNSWORTH

From the Department of Biology as Applied to Medicine,
The Middlesex Hospital Medical School, London

### SUMMARY

A quantitative measure of the proportionality of the pattern of cell differentiation is obtained by separating populations of fruiting bodies into stalks and spores and determining the ratio of their dry weights.

The effect of incubation temperature on the proportion of a population which becomes stalk cells is determined.

The time of determination of this proportion is then indicated by the time in the developmental sequence at which a temperature shift fails to alter it.

The results show that the temperatures of growth, aggregation and migration have no effect on the pattern of differentiation and that temperature alterations during early culmination alter the pattern of differentiation.

This result demonstrates that the pattern of differentiation is not determined during the migrating slug stage, and it is suggested that the axial inhomogeneities seen in the slug are not directly related to the terminal pattern of differentiation of the fruiting body as has been previously suggested.

### INTRODUCTION

Raper, in his original description of *Dictyostelium discoideum* (1935), remarked on the apparent size invariance of the ratio of stalk volume to total fruiting body volume. It appeared to him that, whatever the size of the fruiting body, the size of the stalk bore a constant relationship to the size of the whole sporophore. Quantitative studies of this phenomenon were made by Bonner & Eldridge (1945), Bonner & Slifkin (1949) and Gregg & Bronsweig (1956). All these experiments were interpreted as supporting Raper’s original assumption; in *D. discoideum* the proportion of a fruiting body which is stalk cells is constant over a large range of total cell numbers. The most striking thing about all these determinations is, however, that the proportionality demonstrated was, to use

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1 Author’s address: Biology Department, University of California, San Diego, P.O. Box 109 La Jolla, Calif. 92037, U.S.A.
the words of Bonner himself almost 30 years later, 'Exceedingly rough!' (Bonner, 1967). The scatter on the graphs obtained was very large, with deviations of over 40% error in one case.

Bonner, in his book (1967), sums up the work outlined above by concluding 'the evidence gathered from these experiments is unequivocal, there is a precise control mechanism'. However, I feel that it appears obvious from critical observation of the majority of this evidence that this statement has little justification. If one looks objectively at the published results the doubt could be, not about the precision of the control mechanism, but whether there is any control at all. The reason for such exaggerated claims for the precision of the control of proportions lies, I think, in the confusion of the control mechanisms for the proportioning of differentiation and those of regulation. *D. discoideum* is able to control the proportion of a cell mass which differentiates into each tissue type; irrespective of the total number of cells involved, the ratio of stalk to spore cells is to some degree constant. *D. discoideum* is also able to regulate, to re-establish this ratio to give a normal pattern of differentiation if parts are removed from the cell mass at any time prior to 'determination' when each cell's developmental fate is irreversibly fixed. The undoubtable evidence for the extensive regulative capacities of the slime mould over a very large range of total cell mass appears to have been used to support the idea that this regulation is done with great precision within any one cell mass. It does not necessarily follow that if proportions are controlled over large size ranges, they are controlled with precision at each size. Evidence to support this is provided by both Bonner & Slifkin (1949) and Hohl & Raper (1964), who all concluded that the proportion of a cell mass which differentiates into stalk falls as the cell mass increases in size.

However, it is important as an aid to elucidating the mechanism by which the spatial pattern of differentiation is controlled, to have accurate information on the precision with which, and the time when the pattern is specified (Wolpert, 1971).

Since the work of Raper (1940) had quite elegantly produced the fate map which showed that the cells at the front of a slug formed the stalk it was proposed by several authors that it was a cell's axial position which directed its differential fate. A second hypothesis was then based on this first one, suggesting that the cells in the migrating slug have positional information and have had a pre-pattern imposed upon them such that they begin to differentiate into 'prestalk' and 'prespore' cells in anticipation of their terminal cytodifferentiations (Bonner, 1952; Takeuchi & Sato, 1965).

It has also been suggested, as an alternate patterning mechanism, that any differentiation of the cells in the slug is not of such a nature as to cause them to sort out from each other and does not reflect or direct their fate in the patterns of terminal cytodifferentiation. Rather the spatial pattern of the two cell types seen in the fruiting body is inherent in, and a consequence of, the geometry of
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the morphogenetic changes of culmination, requiring no signalling and no prepatterning (Farnsworth & Wolpert, 1971; Farnsworth, 1973, 1974).

Information on the time of the specification of the final pattern of differentiation is thus important in deciding how the pattern is specified, as this data would elucidate the developmental significance of the axial inhomogeneities seen in the slug which are presently used as evidence of prepattern formation.

MATERIALS AND METHODS

Since the published estimates of the ratio of stalk to spore cells have a wide variation for determinations on individual fruiting bodies, the proportion of each cell type in large populations of fruiting bodies was determined by separating the two on the basis of size and measuring their total dry weights. This technique is restricted to determinations of parameters of the pattern of differentiation of populations of fruiting bodies, not individuals, and does not relate the variance of the ratio of stalk to spore to cell mass as other authors have attempted. However, it does provide a very accurate quantitative measure of a pattern of differentiation; since the deviations are much smaller than those of determinations of individual fruiting bodies it seems that the wide variation in earlier results was due to experimental error rather than a biological variation. A determination of a parameter for a population of fruiting bodies is necessarily a reflection of this parameter for all the individual fruiting bodies and the results are thus comparable with earlier work and can be used to demonstrate accurately differences in the patterns of differentiation in populations formed under different conditions. The two cell types were separated and quantified by the following technique.

Fruiting bodies were allowed to form on freshly prepared 9 cm Petri dishes of 2 % agar. To each plate:

(i) add 5 ml of 0.05 % E.D.T.A., pH 7.4 and sonicate for 15 sec to loosen fruiting bodies.

(ii) Add another 5 ml 0.05 % E.D.T.A. and sonicate for 15 sec to separate the cells.

(iii) Pipette the suspension through six layers of nylon net with a mesh size of 50 μm into a vessel whose dry weight has been determined.

Repeat (i) to (iii) four more times for each plate. Almost all the cells are removed from the plate in the first two washes, the other three serving to wash the spores through the matt of stalks left in the nylon filter.

(iv) Transfer the filter from above the vessel of spores suspension (now 50 ml) to another dried weighed dish.

(v) Dry the vessels for 12 h at 60 °C, 12 h at 80 °C, 36 h at 105 °C until at constant weight.

(vi) The vessels are then cooled in a desiccator and re-weighed. In this fashion, the mean ratio of the dry weight of stalk to spore can be determined for many thousands of fruiting bodies.
The efficiency of recovery was determined by taking a suspension of spores of a known concentration and putting a fixed volume onto an agar plate. The plate was then subjected to the harvesting and separation procedures and the final weight of spores which was recovered was compared with the dry weight of a similar volume of the same spore suspension which had been placed directly into a weighed vessel for gravimetric determination. The efficiency of recovery of the technique was estimated by this method as over 95% in all cases.

The efficiency of separation was determined by plating out the presumed stalk fraction onto a nutrient agar together with *E. coli*, whereupon any contamination by spores resulted in growth of slime mould within 3 days incubation. Each spore suspension was examined with a binocular microscope for the presence of stalks which were easily seen if present. Separation of cell types was judged complete by these methods.

**RESULTS**

(i) *The relationship between the total dry weights of stalk and spore in each of a series of populations of fruiting bodies*

The above technique was performed on about 150 separate cultures of fruiting bodies formed at 22 °C. One can derive an estimate for the mean ratio of the dry weights of stalk to spore for a population of fruiting bodies formed at 22 °C. This can be most usefully expressed as the percentage of the total dry weight which is stalk, and more usefully, the figures can be examined by statistical methods to give confidence limits for such a figure. From my results the proportion of stalk in a population of fruiting bodies formed at 22 °C is 16.6% with a standard deviation of 0.33. There is a 99% probability that a population of fruiting bodies which has, by this method, between 15.6 and 17.6% stalk cells, is from this same (22 °C) distribution. This corresponds reasonably well with the figures of earlier workers which ranged from 12 to 20% for comparable cultures.

(ii) *The variation with temperature of the relationship between the total dry weight of spores and stalks in populations of fruiting bodies*

Cultures of *Dictyostelium discoideum* were grown and allowed to culminate at three temperatures: 18, 22 and 27 °C. The cultures were then subjected to the determination of the relative abundance of the two cell types in populations from each temperature.

Fig. 1 shows the graphs of these results for determinations on more than 100 separate populations at each temperature. As earlier workers have reported the ratio of stalk to spore is altered with culture incubation temperature, the two 5 °C intervals producing three distinct populations, the statistical significance of the differences between the figures was calculated and there is at least a 99.9% chance that the three populations are different. One can thus draw up a range of expected values for each temperature, and the probability of their occurrence.
Fig. 1. The percentage of the dry weight of populations of fruiting bodies estimated as stalk and its variation with temperature. The determination is described in the text. Each point is the mean of three populations. The bar lines mark the 99% confidence limits of the mean ± 3 standard deviations, and the mean itself.

(a) Ninety-nine per cent of all the populations of fruiting bodies formed at 18 °C will have between 17.7 and 21.7% stalk by dry weight.

(b) Ninety-nine per cent of all the populations of fruiting bodies formed at 22 °C will have between 15.6 and 17.6% stalk.

(c) Ninety-nine per cent of all the populations of fruiting bodies formed at 27 °C will be between 12.2 and 13.0% stalk.

These results can be compared with the published data of Bonner & Slifkin (1949). Using the means and standard deviations given by them it is possible
to establish 99% (± 3 standard deviations) confidence limits. Their data give
the following means and 99% probability limits for the percentage of cells
forming stalks: (1) at 27 °C a mean of 2.2 with a range of 0–12.4, (2) at 22 °C
a mean of 14.1 with a range 0–22.94, (3) at 17 °C a mean of 24.1 with a range of
10.5–27.5.

The trend in the means suggests an increase in stalk cell proportion with
decreasing temperature. However, the statistical significance of the data is so
low as to make quantitative comparison with my results of little purpose.

My results demonstrate a linear relationship between stalk proportion and
temperature on a dry-weight basis average over many populations. One can thus
establish firm limits for definition of the quantitative effect of temperature on
the pattern of differentiation as a basis for the interpretation of experiments
involving temperature shifts during morphogenesis. This will enable one to
assign the relative contributions to the final ratio of stalk to spore, of each stage
of development.

(iii) *The time of determination of the ratio of stalk to spore in populations of fruiting bodies*

Having established the limits of the temperature effect it is a simple experi-
mental procedure to assess the time at which the ratio of the two cell types is
‘determined’ in the classical sense.

Cultures were incubated at a given temperature and then transferred to other
temperatures at succeedingly later stages in the morphogenetic sequence. The
transfers were done at increasingly later stages until the final ratio of cell types
differed from that normally seen in fruiting bodies allowed to form completely
at the second incubation temperature, indicating that the pattern had been
determined completely during the initial incubation.

Tables 1 and 2 show, however, the stage of determination does not occur
until very late in development. In fact, even during the terminal culminating
stages, the pattern can still be altered. The slime mould thus does not show
irreversible ‘determination’; the pattern of differentiation is thus not specified
during the developmental sequence, but arises during fruiting body form-
ation.

An extreme demonstration of the failure of the slug stage to influence the
final pattern can easily be made. If slugs are allowed to form at 18 °C in low
ionic environments they will migrate in the dark for extended periods. If, even
after 72 h of migration at 18 °C, the lid of the dish is removed for 5 min and the
culture exposed to overhead light, culmination will be initiated. If such cultures
are then left at 18 °C for another hour and then transferred to 22 °C for the
last 4 h of culmination, fruiting bodies will be formed which have a proportion
of stalk cells identical to that of fruiting bodies produced entirely at 22 °C. If
the slug has not the courage of its ‘pre-pattern’ convictions after 73 h, and the
pattern can be totally dependent upon the last 4 h of development, the pre-pattern,
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Table 1. Cultures were grown at 18 °C and transferred to 22 °C after various times giving the following alterations in the percentage of cells which form stalk.

<table>
<thead>
<tr>
<th>Period of incubation at 18 °C before transfer to 22 °C (h)</th>
<th>Stalk %</th>
<th>99 % confidence limits</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>16-6</td>
<td>15-6-17-6</td>
</tr>
<tr>
<td>2</td>
<td>16-1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>16-4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16-9</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>15-2</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>16-4</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>21</td>
<td>18-4</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>19-8</td>
<td></td>
</tr>
<tr>
<td>24 or more</td>
<td>19-7</td>
<td>17-7-21-7</td>
</tr>
</tbody>
</table>

Not until after 20 h, when the fruiting body is forming, does transfer to 22 °C produce a population of fruiting bodies with a proportion of stalk not characteristic of those grown completely at 18 °C.

* Line indicates the cut-off where the measured value exceeds the value expected for incubation at 22 °C and becomes that normal for 18 °C populations.

Table 2. Experiments such as those described in Fig. 1 for a variety of temperature changes

<table>
<thead>
<tr>
<th>Temperature of growth (°C)</th>
<th>Temperature of migration (°C)</th>
<th>Temperature of culmination (°C)</th>
<th>% of the dry weight of the population isolatable as stalks</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>No. samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>18</td>
<td>18</td>
<td>19-7</td>
<td>0-67</td>
<td>148</td>
<td></td>
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<tr>
<td>22</td>
<td>22</td>
<td>22</td>
<td>16-6</td>
<td>0-33</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>27</td>
<td>27</td>
<td>12-6</td>
<td>0-13</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>18</td>
<td>27</td>
<td>12-9</td>
<td>0-47</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>22</td>
<td>22</td>
<td>15-8</td>
<td>0-21</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>22</td>
<td>18</td>
<td>20-1</td>
<td>0-41</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>22</td>
<td>27</td>
<td>13-3</td>
<td>0-29</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

The figures show that the temperatures of growth, aggregation and migration have no effect on the proportion of stalk in a population. The portion of stalk is dependent solely on the temperature of culmination.

if it exists as such, is of little developmental significance in the determination of pattern.

This conclusion would appear to present a paradox in the light of the consideration of many authors that the differences between the front and rear of a slug represent some differentiated pattern of ‘tendency to form’ stalk and spore (Bonner, 1952). If this is true, the observation that the pattern can be altered
during the final 4 h of culmination raises the question of why there is such a labile pre-pattern in the slug at all. There appears to be evidence that there are axial inhomogeneities of cellular properties in migrating slugs (Newell, Ellingson & Sussman, 1969; Farnsworth & Loomis, 1974). However, there is no good evidence which directly links such inhomogeneities to the pattern of differentiation seen in fruiting body construction. It may well be that the differential fate of a cell is determined by its position though this work would suggest that such a determination does not occur in the migrating slug, since properties of the slugs' development at 18 °C are not reflected during fruiting body construction at 22 °C. It therefore seems that even if there is a potential system for generating positional information in the migrating slug that the cells are not directed in some spatially organized and correlated fashion to embark upon pathways of differentiations directed towards stalk or spore formation during migration. Even if cells do behave in such a fashion, it is not at this stage that the proportionality of the pattern of differentiation is specified or regulated. When one also considers that a migrating slug is not required for the formation of normal fruiting bodies, one might conjecture that the axial inhomogeneities of the slug reflect not a pre-determination of pattern, but are rather a manifestation of the constitutive polarity of the slug or its integral coordination of movement.

The results reported above lend considerable support to previous work of the author (Farnsworth, 1973, 1974) in suggesting that the pattern of differentiation is specified during culmination and that the migrating slug is of ecological rather than developmental significance. It appears that a comprehension of the manner in which the ratio of stalk to spore is determined may be found within studies on the morphogenetic changes of culmination rather than in studies on axial inhomogeneities within the slug.

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REFERENCES

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