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Tip regeneration and positional information in the slug of *Dictyostelium discoideum*

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SUMMARY

We show in this paper that in the case of the slug of the cellular slime mould *Dictyostelium discoideum* the time which it takes for a new tip to regenerate at a given level can be used as a measure of positional information at that level. Our basic experiment consists of amputating slugs at various distances from the existing tip and thereby inducing the regeneration of a fresh tip; the time needed for regeneration is estimated by two independent methods. An identical operation, when performed in the anterior portion of a previously cut slug, tells us how this position-dependent regeneration time adjusts to a sudden change in the size of the slug. The reasons which lead us to conclude that tip regeneration times are in one-to-one correspondence with positional information are as follows. (i) Depending on their positions, the cells in a slug take different times to regenerate a new tip following amputation; (ii) regeneration times are scaled in relation to the total length of the slug and increase monotonically with the length cut off; and (iii) as judged by the regeneration time, cells can assess and remember their positions relative to the length of the slug. Our results highlight the importance of two rate processes. One might think of the slower process as being related to the setting up of a system of positional information in the slug, and the faster process as being a reflection of the kinetics of the positional value changing locally till it reaches the level appropriate to a tip.

INTRODUCTION

If simple rules can be used to describe the emergence of spatial order in developing systems, it is important that such rules be expressed quantitatively. The rule we have in mind here is that in a regulative organism, the future fate of cells in the embryo depends only on their relative positions within it (Driesch; quoted in Wilson, 1925). To rephrase this in more contemporary language, the system of positional information in a regulative embryo scales with respect to total size (Wolpert, 1971). We have attempted to study this rule by performing experiments on tip regeneration in the slug of the cellular slime mould *Dictyostelium discoideum*. The slug, which is formed by the aggregation of single amoebae, is the equivalent of an embryonic stage in *Dictyostelium* (Bonner, 1967). In the course of time the cells comprising the slug differentiate into one

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of the two (or possibly three) cell types that constitute the final structure, the fruiting body. Studies on the pattern of this differentiation suggest both that an initial predisposition to form one or another cell type exists (Takeuchi, 1969; Leach, Ashworth & Garrod, 1973; Forman & Garrod, 1977), and that the fate of the cells in the slug is strongly conditioned by their relative positioning within the cell mass (Raper, 1940). We do not discuss this question here except to state that the fact of pattern regulation (Sakai, 1973) is in itself sufficient to show that a cell's position influences its fate; Tsang & Bradbury (1981) have recently provided further evidence favouring such a conclusion. Therefore one can meaningfully talk of positional information in the slime mould slug, and one can speak of the slug as a regulative organism in the traditional sense of embryology. Since the slug is also approximately one dimensional in appearance, with a length typically 10 to 20 times the width, the hypothesis of regulation can be stated in the following simple form (Wolpert, 1971; Robertson & Cohen, 1972): the fate of a cell at location x – or, the positional information at x – depends on the ratio x/L where L is the total length of the slug and x is measured from one of its ends. Our interest, ultimately, is in understanding the basis of this dependence on x/L.

Clearly, as the first step towards such an understanding it has to be established that there is something in the slug itself, prior to the onset of differentiation, that varies with relative position and so (conceivably) is correlated with positional information. We have done this by making use of the result that the tip, a specialised morphological structure at one end of the slug (the 'anterior'), regenerates spontaneously when cut off (Raper, 1940). Regeneration is due to some of the cells in the remaining tipless fragment taking over the appearance and function of a tip ('function' here refers to the organizing ability of the tip both in the slug as well as at later stages (Raper, 1940; Rubin & Robertson, 1975), similar to that of the organizer in amphibian embryos (Spemann, 1938) or the hypostome in *Hydra* (Browne, 1909, cited in Webster, 1971)). We found that the time τ_R needed for a new tip to regenerate depended only on the relative position x/L of the cut (Lokeshwar & Nanjundiah, 1981). This suggests that the regeneration time itself can be used as a measure of positional information along the slug, because (i) it is a property of the cells in the slug before the onset of overt differentiation; (ii) it has a bearing on the specification of pattern in the slug, inasmuch as the tip is an organizer; and (iii) it scales with the total size of the slug, that is, τ_R is a function of the ratio x/L mentioned earlier.

If such a suggestion is valid, and if the slug is indeed a regulative embryo, it must be possible to demonstrate that the regeneration time regulates if the size of the slug is changed. Here we attempt to provide a demonstration. Our method is as follows. To begin with, we extend our earlier findings and show that regeneration times vary linearly with relative levels of cutting: τ_R is a linear function of x/L. Next, we ask whether this relationship is permanent; in other words, once a slug is formed, is the system of positional information in it fixed once and for all? To get an answer we perform a second amputation, now on one

of the small slugs resulting after cutting a larger one, and look at the time needed for a tip to regenerate at the second cut surface. It turns out that as judged by the time taken to regenerate a new tip, the cells in the daughter slug carry with them a slowly decaying memory of their previous locations in the parent. After a sufficient period elapses this memory gets erased and a new tip forms in a time appropriate to the position of the cut surface in the daughter slug. Therefore the time needed to regenerate a tip does regulate. We end with a discussion of the implications of our findings for theories of pattern formation in the slime mould slug.

METHODS

Exponentially growing cells of *D. discoideum* NC4-H were washed free of bacteria and induced to aggregate by being spread at a density of $7 \pm 1 \times 10^5$ cells/cm^2 on petri dishes containing 2 % water-agar; after spreading the plates were incubated in the dark at 21 °C–22 °C. The slugs used later ranged in age from 4 h to 60 h following the end of aggregation. Amputation and grafting (Figs 1A, 1B) were performed under a dissecting microscope using a small scalpel, and the slugs were monitored at regular intervals thereafter. The temperature was maintained at 24 ± 1 °C during experimentation. The donor and recipient slugs in a graft were of about the same size as judged by a scale in the eyepiece; also, donor fragments were marked by prestaining cells with neutral red (Bonner, 1952). Care was taken to see that the grafted tip, though inevitably of a larger size in bigger slugs, was of a fixed size when removed from slugs of a given length. In no case did the length of the transplanted region exceed 10 % of that of the donor slug. The slug length L varied between experiments from 0·75 mm to 2 mm. Second amputations were performed (in a separate set of experiments) on anterior slug fragments at various times after the first amputation; this is indicated in Fig. 1C. Anterior fragments were used because posterior fragments commonly round up after one cut and proceed to erect themselves soon after a tip regenerates (Raper, 1940).

RESULTS

We club together results referring to the same relative level of amputation (x/L) since all times of interest to us scale with the overall length of the slug (Lokeshwar & Nanjundiah, 1981). Also, the results are the same for slugs aged between approximately 4 h and 60 h from the time they are formed (Table 1). Defining the regeneration time τ_R as that time by which a tip has regenerated in 50 % of the cases, we find that τ_R increases with the length of the fragment cut (Fig. 2A). The dependence is linear (Fig. 3), and τ_R varies from 10·0 min (x/L 5 % to 10 %) to 97·5 min (x/L 60 % to 70 %) within the range of values for x/L

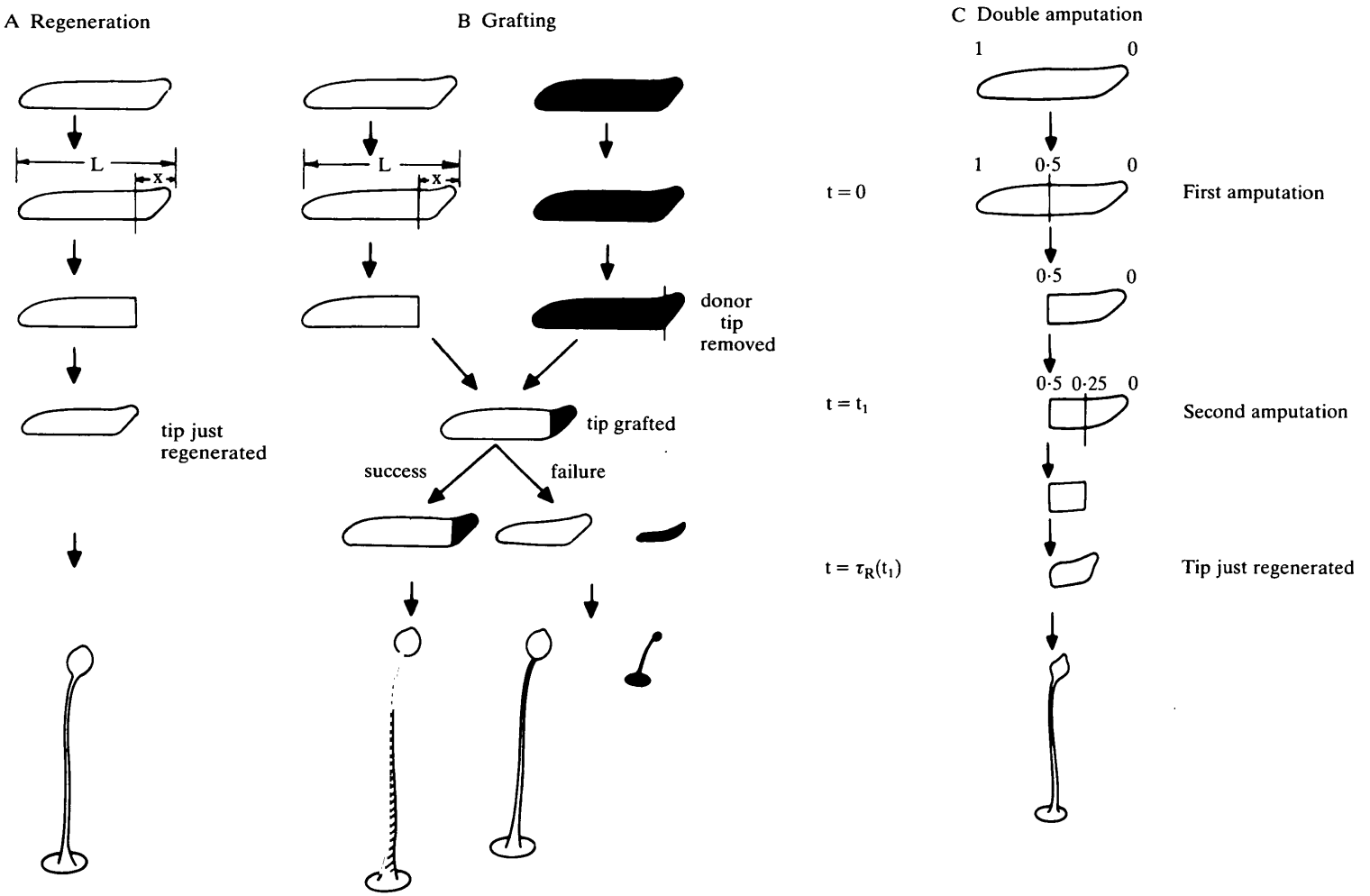


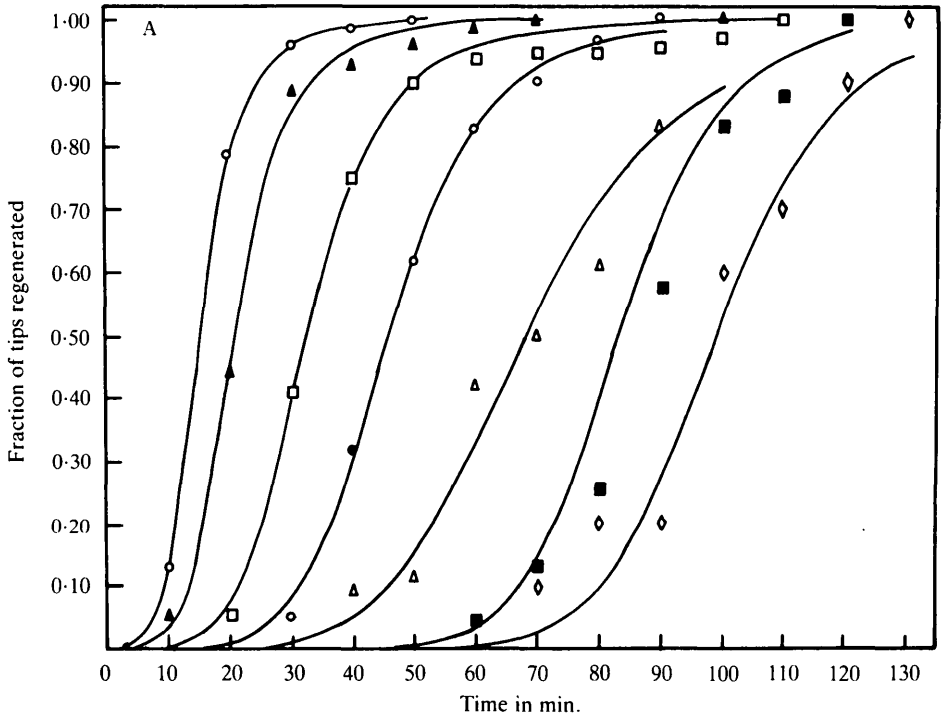
Fig. 1. Schematic representation of experiments pertaining to regeneration (A), tip grafting (B) and double amputation (C). See text for details.

used by us. From Fig. 2B, at a fixed x/L the success of a tip-grafting experiment decreases with time in a manner which mirrors the regeneration of a new tip. Further, the probability that a graft is successful increases with increasing posterior levels in the host (Fig. 2(b)), confirming the observation made by Durston (1976). Again, for a given x/L we estimate the time τ_G at which a tip graft is rejected in one-half of the number of attempts. τ_G and τ_R are more or less the same; this shows that our estimates of regeneration times have an objective basis (Fig. 3). The double-amputation experiments (Fig. 1(c), Fig. 4) show that even though τ_R normally depends only on the relative level at which the cut is made, its value can be affected by an abrupt change in relative positions. A second cut made immediately after the first elicits a response characteristic of the position of the cut surface in the 'old' slug. As the time between the two cuts is increased, regeneration times also gradually increase till they reach a value appropriate to the level of the cut surface in the 'new' slug.

DISCUSSION

Our basic results may be summarised as follows. First, there is a definite time needed for a new tip to regenerate after an old one is removed; this time depends on the *relative* position along the slug at which regeneration occurs, and it increases linearly with increasing distance from the tip (anterior→posterior; Figs 2(a), 2(b), 3). Secondly, if the relative position of cells in the slug is changed by cutting the slug in two – a process which may be compared to separating the blastomeres in an embryo – the cells need some time before they can respond to a further cut in a manner appropriate to their new positions (Fig. 4).

In analysing the results, we feel that rather than trying to force our findings into a theoretical model, in the present state of our understanding it is more worthwhile to see what we can infer from them. The basic assumption we shall make is that the set of regeneration times τ_R is in one-to-one correspondence with positional information in the slug. To justify this, two other reasons can now be added to those given earlier. For one thing, the very fact that we obtain comparable quantitative results with slugs of widely differing ages and sizes (Table 1) indicates that we are looking at an aspect of pattern which is both stable in time and invariant in form from slug to slug – precisely what one would demand of a system of positional information. The second reason, of course, is that the regeneration times regulate in response to a change in the size of the slug (Fig. 4 and unpublished results). Needless to say, though positional information conditions future cell type, and both vary from cell to cell, one should not expect that the manner in which they do so is identical (Wolpert, 1971); a smooth gradient of positional information can be used to generate a discrete array of cell types. In our case, even though a boundary in the slug separates future stalk and spore cells (Bonner, 1967) the positional information does not change abruptly anywhere.


A
Fraction of tips regenerated
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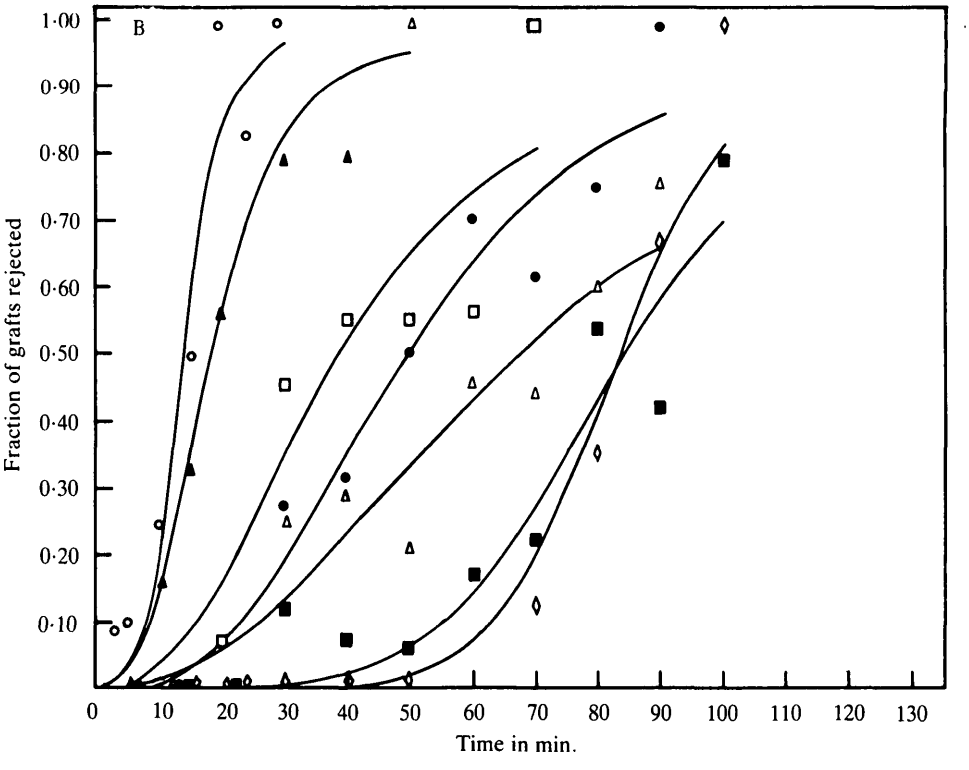
Time in min.


B
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Time in min.

Table 1.

Evidence that regeneration times are only weakly dependent, if at all, on slug age. The age as defined here is the time following the completion of aggregation. Since it takes typically 4 h for the migrating slug phenotype to first appear under our conditions, the minimum slug age cannot be very much smaller. Numbers in the last column of the table were obtained after performing a linear regression of mean regeneration times on slug age (all correlation coefficients > 0·95). The number of experiments analysed in the compilation of this table is somewhat smaller than the number used in the rest of the paper, because cases with only one slug of a given age had to be ignored here. This leads to minor discrepancies: for instance, in the 5 %–10 % case, one experiment, consisting of a single 3 h-old slug which regenerated a tip in 20 min, is not included here. Note that experimentally observed regeneration times (col. 2) are used here, and not the times obtained by analysis of data (Fig. 2a, 3a).

Within each range of lengths cut, we have verified the hypothesis that the various mean regeneration times for slugs of different ages are all equal to the population mean regeneration time, i.e. the regeneration time averaged over slugs of all ages (χ^2 test, P rejection < 0·005 in every case). This is equivalent to saying that the regeneration times are age independent. To take a specific example, in the case of cuts within 21 % to 30 %, the relation between slug age and regeneration time is as follows:

6 h: 38·7 ± 7·5 min; 12 h: 32·8 ± 8·6 min;
24 h: 44·5 ± 7·4 min; 36 h: 41·3 ± 7·0 min;
48 h: 59·0 ± 9·4 min; 53 h: 35·0 ± 8·7 min.

Percentage cut $\frac{x}{L} \times 100$ (No. of cases)	Regeneration time in min., mean ± S.D.	Slug age in h		Ratio of maximum age to minimum age	Maximum ratio of regeneration times
		minimum	maximum		
5–10 (45)	18·3 ± 6·9	8	60	7·5	1·01
11–20 (94)	23·7 ± 9·8	4	60	15·0	0·95
21–30 (75)	37·2 ± 14·0	6	53	8·8	1·56
31–40 (75)	51·2 ± 14·3	4	48	12·0	1·38
41–50 (33)	73·3 ± 19·7	4	48	12·0	1·41
51–60 (32)	87·7 ± 14·1	4	48	12·0	1·08
61–70 (11)	105·0 ± 10·0	4	40	10·0	1·02

Fig. 2. Kinetics of tip regeneration (A) and graft acceptance (B). Abscissa, time after amputation (t) in minutes; ordinate, fraction of cases (f) successfully regenerated by that time (A), and fraction of tip grafts performed at that time which were failures (B). The symbols refer to the percentage length cut ($x/L \times 100$, Fig. 1): ○ (5 %–10 %), ▲ (11 %–20 %), □ (21 %–30 %), ● (31 %–40 %), △ (41 %–50 %), ■ (51 %–60 %), ◇ (61 %–70 %). Only mean values are plotted, from an average of 52 experiments in each case. Error bars have not been shown for the sake of clarity; to take an example, in the regeneration experiments, the 31 %–40 % points actually refer to a mean of 36 % (S.D. 3·3 %) from 77 cases, and the observed mean regeneration time was 51·3 min (S.D. 13·9 min). The smooth curves are obtained by assuming sigmoidal relationships between f and t; the curves represent least-squares fits. τ_R is that time at which one-half of the amputated slugs belonging to a given class (x/L) have regenerated a new tip; τ_G is that time of grafting at which 50 % of the grafts in a given class are unsuccessful. The results pertaining to grafting show a much wider scatter than those referring to regeneration. In our theoretical curves (Fig. 2B), this has the consequence that one curve sometimes intersects another. Nevertheless, the time at which a graft is successful in one-half of the cases is the same whether estimated from the theoretical curves or directly from the experimental points.

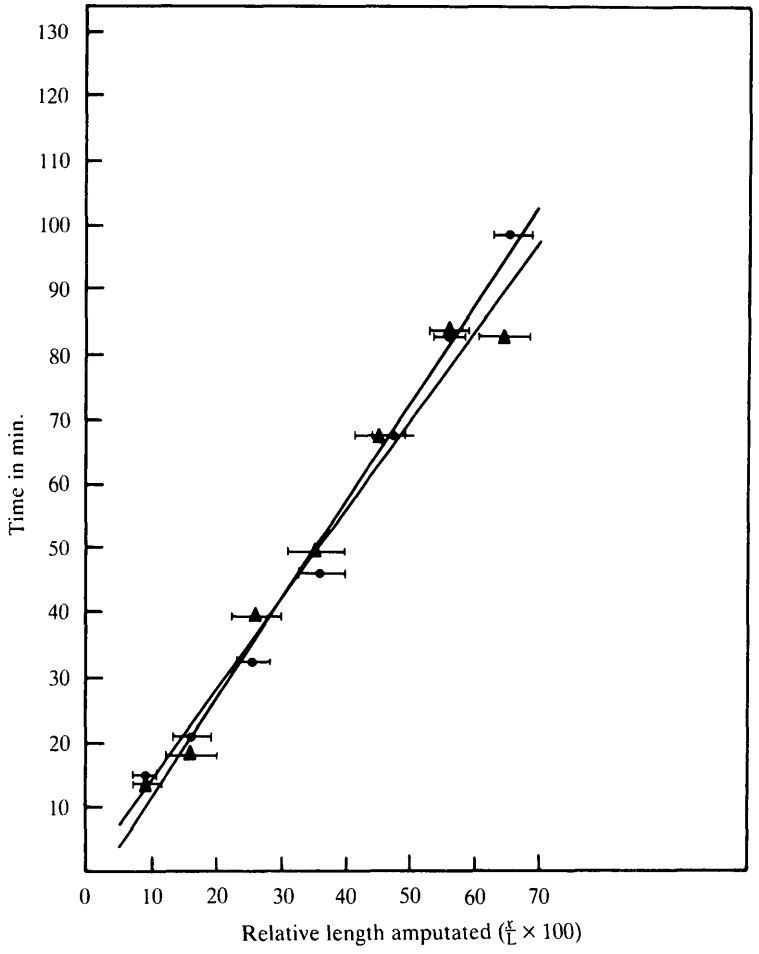

Fig. 3. Variation of (●) τ_R with $x/L \times 100$ and (▲) τ_G with $x/L \times 100$ (Figs 1, 2). Abscissae, percentages cut; ordinates, time in minutes. The horizontal bars refer to one standard deviation. Values of τ_R and τ_G are obtained from curve-fitting as explained in Fig. 2. The straight lines are obtained by linear regression (correlation coefficient >0·99). Inspection shows that for a given x/L, τ_R and τ_G are practically identical. The fitted curve (●) is $\tau_R = \tau x/L + c = \tau - x'/V + c$ where the 'time constant' $\tau = 153{\cdot}4$ min, $x' = L - x$ length of remaining fragment (Fig. 1), $c = -3{\cdot}6$ min and is negligible, and the 'speed constant' V equals L/τ and therefore increases with slug length. If the data are grouped into different classes according to slug lengths, V varies from about 6 μm/min for the smallest slugs to 11 μm/min for the largest ones.

In trying to explain the dependence of regeneration time on relative position, two alternative hypotheses suggest themselves. These are that tip regeneration is a consequence of (A) purely local phenomena occurring at the cut surface, or (B) a cooperative decision taken by all the cells remaining in the posterior fragment. According to hypothesis (A) the observed regeneration time τ_R is a function only of the position of the cut; so it is due to some property permanently (or irreversibly) resident in the cells at the cut surface. Graft rejection clearly

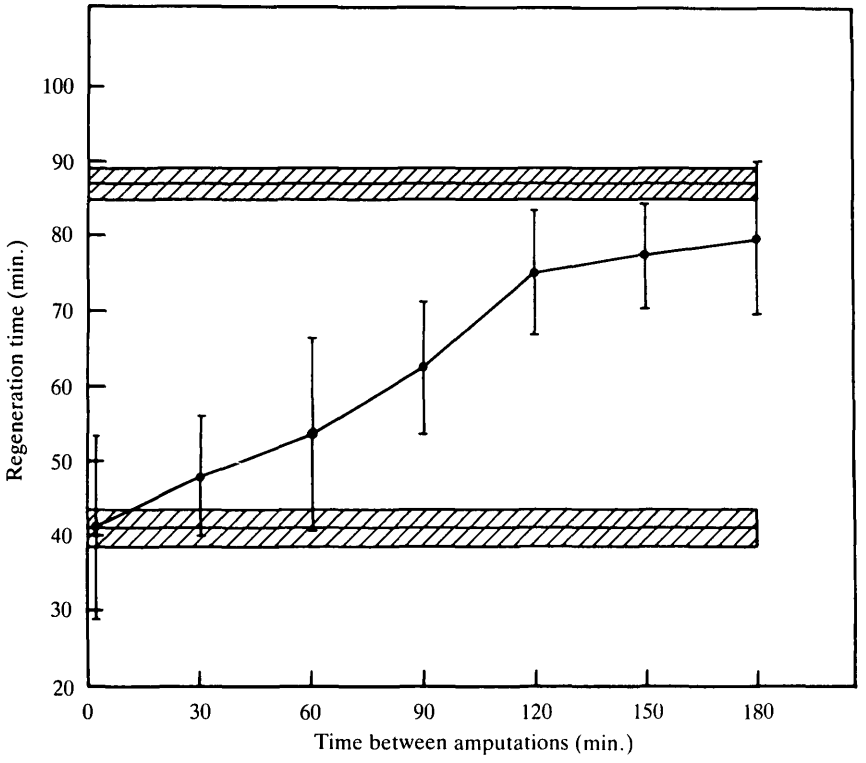

Fig. 4. Double amputation experiments. Abscissa, time between first and second amputation, and ordinate, regeneration time; both in minutes. Each point on the curve represents an average of about 15 experiments (see Fig. 1C) and the vertical bars stand for ±1 S.D. The horizontal bands represent regeneration times (±S.E.M.) for 25% and 50% amputation levels after a single cut (as in Fig. 1A). These regeneration times agree with those listed in Table 1. The curve shows that when a second amputation is performed in the anterior fragment immediately after the first ($t_1 = 0$, Fig. 1C), the time needed for a new tip to regenerate is what it would have been if only the second cut had been made at the same position ($x/L = 25\%$, Fig. 3) and in the entire ('old') slug. As the time t_1 at which the second cut is made increases, the time for regeneration ($\tau_R(t_1)$, Fig. 1C) correspondingly increases until it reaches the value appropriate to only the second cut being made in the fragmented ('new') slug ($x/L = 50\%$, Fig. 3).

depends on whether a new tip has already regenerated or not (and so differs from the corresponding case in *Hydra*; see Wolpert, Clarke & Hornbruch, 1972).

The double-amputation experiments were performed in an attempt to distinguish between alternatives (A) and (B). Ideally, one would have liked to make the second amputation in the posterior fragment after it had regenerated a new tip (or even during the course of regeneration); however, the rounding up which frequently follows tip removal made this impractical. We have instead performed the second cut (at varying times after the first) in the anterior fragment (Fig. 1C). The predictions are straightforward. According to hypothesis (A), the

regeneration of a new tip should occur after a time which is appropriate to a level of 25 %, that is, in accordance with relative position in the 'old' slug. On the other hand, if hypothesis (B) is correct, the regeneration time should finally scale with respect to position in the 'new' slug and thus be appropriate to a level of 50 %. We say 'finally' to take into account the possibility that the second cut is performed so soon after the first one that the cells in the previous anterior fragment have not had a sufficient amount of time to communicate over the entire length of the fragment.

Figure 4 shows the two extreme possibilities (shaded regions) as well as the actual results obtained. These results clearly argue against an irreversible determination of regeneration times but rather suggest that the time needed for regeneration at a cut surface is conditioned by mutual communication amongst all the cells in the slug, or at any rate amongst cells other than just those which lie at the cut surface. So even if a tip regenerates on account of the local chemistry (say) at the cut surface, how long it takes to do so depends on a communal decision of the slug's cells. In terms of positional information, we may say that the (relatively slow) time course over which the regeneration time increases from the lower shaded region to the upper one in Fig. 4 is the same as the time course over which the pattern of positional information in the anterior fragment regulates so as to resemble that which prevailed in the intact slug. Once again, we have an indication of the inherent stability (or inertia, or homeostasis) of positional information in the slug; this is reminiscent of comparable observations in other systems. For instance, in referring to *Amphioxus*, in which a single early blastomere can give rise to an entire adult, Wilson (1900; p. 419) says '. . . it is a very interesting fact that the isolated blastomeres of *Amphioxus* sometimes show, in the early stages of cleavage, peculiarities of development that recall their behaviour when forming part of an entire embryo'. In the chick limb bud, the influence of a grafted zone of polarising activity (which is an organizer in limb development) is remembered by responding cells even after the graft has been removed or rendered ineffective; a phenomenon aptly termed 'positional memory' by Smith (1979). Similarly, in studying the regulation of the visual projection onto a halved optic tectum in the goldfish, Yoon (1976) finds that a compressed-but-normal projection does result, but only over a course of time and after an uncompressed projection – corresponding to what we might call 'old' coordinates – has first formed.

Our findings thus indicate the existence of a stable, monotonic, regulative gradient of positional information in the slug, the tip being at a maximum (or minimum) of the gradient. As to how this gradient is generated, we have no certain knowledge. An interesting suggestion which has been made is that the slime sheath, which surrounds the slug and has a uniformly increasing thickness from anterior to posterior, could provide positional information by acting as a diffusional barrier to appropriate morphogens (Loomis, 1972; Farnsworth & Loomis, 1974, 1975; Sussman & Schindler, 1978). While this is plausible, it does

not solve the problem of how the gradient regulates, because the gradient in slime sheath thickness is the same from slug to slug rather than being scaled with respect to slug length.

If and when a satisfactory theory does predict our results, the theory will have to account for one – and perhaps two – numerical constants. The first is the 'time constant' τ of about 2·5 h (Fig. 3); it is the maximum time needed for a tip to regenerate (if we are correct in extrapolating the observed linear behaviour up to a cut of 100 %), and is expected to be observed when all but the very hindmost portion of the slug is cut off. The other constant, which may or may not be fundamental, is the value of V, ranging from 6 μm/min to 11 μm/min, which we have called the 'speed constant' (Fig. 3). Something else that a theory will have to explain concerns the basis of positional memory in the slug. All we can say is that the order of time needed for this re-adjustment of positional information is certainly more than that needed for tip regeneration at a comparable position (Figs 3 and 4). At the other extreme, we have the time needed for a change of the determined state in anterior slug fragments (Sakai, 1973); this varies from 3 to 7 h and is therefore much larger than the approximately 2 h which it takes for positional information to get accommodated into the reduced size of the slug (Fig. 4). Based on this difference in times we would like to suggest that tip amputation sets into motion the following sequence of concurrent events (perhaps, though not necessarily, in serial order): first, tip regeneration; next, respecification of positional information; and lastly, an initiation of genetic and biochemical changes which mirror what happens at the end of aggregation and result in a pattern of differentiation into stalk and spore cells. Interference with any of these events would be expected to disturb the final stages of normal morphogenesis in *Dictyostelium*.

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