Biochemical and Cytological Heterogeneity of the Differentiating Cells of the Cellular Slime Mould *Dictyostelium discoideum*

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1. The slug stage of the cellular slime mould *Dictyostelium discoideum* has been shown to contain two types of cell, which differ in buoyant density. 2. These two cell types also differ in cytological appearance and histochemical behaviour and have very different enzymic activities. 3. Evidence is presented suggesting that the lighter of these two cell types corresponds to cells from the posterior region of the slug (pre-spore cells) and the heavier of the two to cells from the anterior region of the slug (pre-stalk cells).

The cellular slime mould *Dictyostelium discoideum* has a complex life cycle (Bonner, 1959). When the food supply is exhausted the hitherto solitary myxamoebae associated on a solid surface to form multicellular and macroscopic aggregates. An aggregate (called a slug, pseudoplasmodium or grex; Plate 1c and 1d) acquires a longitudinal polarity and, after a period of concerted movement, the component cells of the slug differentiate to form one of the two cell types characteristic of the mature fruiting body, namely stalk or spore cells. The anterior cells of the slugs become stalk cells and the posterior cells become spore cells (Raper, 1940). In a suitable environment the spores germinate to form free-living and solitary myxamoebae.

The slug is known to have a definite organization within itself since the anterior and posterior regions can be distinguished histochemically (Miné & Takeuchi, 1967; Takeuchi, 1960). However, this differentiation, unlike the differentiation to form spore and stalk cells, is readily reversible (Raper, 1940).

We now report the isolation, from slugs, of two cell populations that differ in buoyant density, cytological appearance and enzymic composition. We suggest that these two cell populations are derived from the anterior and posterior regions of the slug.

MATERIALS AND METHODS

Preparation and dissociation of slugs. The myxamoebae of *Dictyostelium discoideum* NC4 were grown on nutrient-agar in association with *Aerobacter aerogenes* as described by Sussman (1966). After 40 hr. growth at 23° (when

virtually all the bacteria had been eaten) the myxamoebae were harvested in cold distilled water and plated out on Millipore filter supports as described by Sussman (1966). After 15 hr. incubation at 23° the myxamoebae had formed slugs and these were washed off the filters with 10 mM-tris-HCl buffer, pH 6.7, containing 10 mM-EDTA.

The slugs were collected by centrifugation and resuspended in 10 mM-tris-HCl buffer, pH 6.7, containing 10 mM-EDTA. A concentration of 8×10^8 – 10×10^8 cells (from eight to ten Millipore filters)/ml. of tris-EDTA suspension medium was found to give optimum dissociation and subsequent separation of the cells. Vigorous shaking of this suspension in 1.0 ml. portions at 4° for 10 min. in the presence of glass beads dissociated the slugs and produced a suspension of single cells (Loomis & Sussman, 1966).

Isopycnic centrifugation. Solutions of the sodium salt of 3,5-diacetamido-2,4,6-tri-iodobenzoic acid were prepared by addition of NaOH to suspensions of the free acid prepared by addition of HCl to commercially available Urografin (obtained from Schering Chemicals Ltd., Burgess Hill, Sussex). Linear density gradients (17 ml. total volume) were prepared, by using a conventional gradient-forming device, from solutions of this salt in water at pH 7 of refractive index 1.3800 (ρ 1.194) and refractive index 1.3950 (ρ 1.253). Samples (1–2 ml.) of the cell suspension prepared as described above were layered over the gradient and centrifuged at 52 000g in a 3 × 20 ml. swing-out rotor for 60 min. in an MSE 50 centrifuge. A slight pellet (which did not contain any cells) and three distinct bands were observed (Plate 1a). The lightest of these bands, which formed at the interface between the aqueous layer and the Urografin solution, did not contain any cells and it, and the pellet, represent material derived from cells inevitably broken during dissociation of the slug. The two intermediate bands contained unbroken cells and are labelled heavy and light in Plate 1(a).

The gradients were fractionated by piercing the bottom of the tube, through the pellet, with an MSE tube piercer. The 20-drop fractions were collected. Each band of cells came out in three fractions, which were separated by 20–30 drops. The appropriate fractions were pooled, diluted with

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3 vol. of cold 10mm-tris-HCl buffer, pH 7.6, and kept at 0° for 5 min. The cells were collected by centrifugation and washed once by resuspending in 4 ml. of 10mm-tris-HCl buffer, pH 7.6, and centrifuging again. The final pellets were then suspended in the appropriate media for further analysis.

Enzyme assays. Cell suspensions in equal volumes (5 ml.) of 10mm-tris-HCl buffer, pH 7.6, were subjected to ultrasonic disintegration (1 min. at 4.5 A with the MSE ultrasonic disintegrator) at 4° and the resulting cell-free solutions assayed. Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951), alkaline phosphatase as described by Gezelius (1966), UDP-glucose pyrophosphorylase as described by Ashworth & Sussman (1967), *N*-acetylglucosaminidase as described by Loomis (1969), citrate synthase as described by Srere, Brazil & Gonen (1963) and malate dehydrogenase as described by Bergmeyer (1962). Specific activities are expressed as nmoles of substrate used or product formed/min./mg. of protein.

Examination of cells in the electron microscope. Heavy and light cell fractions were suspended in a solution of 2% glutaraldehyde in 0.2M-sucrose-10mm-tris-HCl buffer, pH 7.6, for 2 hr. at 0°. The fixed cells were collected by centrifugation, post-fixed in buffered 2% osmic acid (30 min.), dehydrated through an ethanol series, infiltrated with propylene oxide and Araldite and polymerized for 2 days at 60°. Silver and grey sections were cut on a LKB Ultratone III and were stained with uranyl acetate [freshly prepared 2% solution in 50% (v/v) ethanol]. The sections were examined in a Siemens Elmiskop IA electron microscope at an accelerating voltage of 80 kv with a 200 μ m. condenser aperture and an electron-optical magnification of 8000-10000.

Histochemical staining of cells in slugs. Miné & Takeuchi (1967) have described a procedure for staining whole slugs so that only the anterior portion of the slug is stained. We have confirmed their observations and slightly modified their procedure to stain partially broken slugs before their dissociation to single cells and centrifugation through Urografin gradients.

Slugs were prepared on ten Millipore filter supports as described above and harvested by washing off with 10mm-tris-HCl buffer, pH 7.6. This slug preparation was centrifuged and the pellet of partially broken slugs was resuspended in 4 ml. of a solution containing 0.1% Nitro Blue Tetrazolium (1 ml.), 0.1M-sodium-potassium phosphate buffer, pH 7.2 (1 ml.), and 0.1M-sodium succinate (2 ml.), preheated to 40°. The suspension was incubated at 40° for 10 min., then centrifuged in the cold and the purple pellet resuspended in 1 ml. of 10mm-tris-HCl buffer, pH 6.7, containing 10mm-EDTA. A 1 min. period of vigorous shaking at 4° in the presence of glass beads was sufficient to produce a suspension of single cells, which was layered on a Urografin gradient and centrifuged as described above.

RESULTS

The general appearance of the slug stage of *D. discoideum* is shown in Plate 1(c) and 1(d).

The procedure described for dissociating the slugs is a more violent modification of that described by Loomis & Sussman (1966). Complete dissociation of the slugs to single cells was found to be essential to obtain clear separation of the heavy

and light cells. Incomplete dissociation resulted in a feathery appearance of the two bands and resulted in incomplete separation, which made fractionation difficult. An inevitable consequence of the necessity to obtain single cells was that 30-50% of the cells present in the slugs were broken during the dissociation procedure. The extent of breakage varied from experiment to experiment, but, since this did not affect the enzyme distribution in the two cell fractions, there is no evidence that some of the cells of the slug are more prone to destruction during the dissociation procedure than are others.

All the cells applied to the gradient were viable, since when plated out with *A. aerogenes* on nutrient-agar all gave rise to clones of myxamoebae that formed fruiting bodies in the normal fashion. Provided that care was taken to wash out the Urografin as described in the Materials and Methods section from the heavy and light cell fractions, all the cells in these fractions similarly gave rise to clones of myxamoebae and normal fruiting bodies when plated out with *A. aerogenes* on nutrient-agar.

When cells from the heavy and the light fractions were resuspended in distilled water and put down on Millipore filter supports at 10⁸ cells/support then normal fruiting bodies were formed from both cell fractions after approx. 20 hr. incubation at 23°. These fruiting bodies, although morphologically normal and containing viable spores, were, however, very much smaller and thus more numerous than those formed from a similar number of mxyamoebae. Similar observations were made if separated cells from dissociated slugs were resuspended in distilled water without centrifugation through Urografin gradients and put down on Millipore filter supports, in agreement with the findings of Loomis & Sussman (1966).

Enzymic activities in heavy and light cell fractions. The specific activity of a number of enzymes varied in the two cell fractions (Table 1). In no case was there evidence of any destruction of enzymic activity during centrifugation, since all the enzymic activity applied to the gradient could (after making allowances for the degree of cell breakage) be accounted for in the two cell fractions. In one case (pyrophosphatase) this was not true and losses of 20-70% of the activity have been observed. Results for this enzyme are not presented since the degree of loss is very variable and unpredictable.

Origin of the two cell types. When slug preparations are treated with the succinate-Nitro Blue Tetrazolium reagent, under conditions in which it is known that only the anterior portion of the slug is stained, and the slugs are then dissociated to single cells that are separated in a Urografin gradient, the gradient has the appearance shown in Plate 1(b). Only the heavy cell band is stained

EXPLANATION OF PLATE 1

Separation of heavy and light cell fractions from the slug stage of *D. discoideum*. (a) Cells prepared from unstained slugs after centrifugation through a Urografin gradient. (b) Cells prepared from fragments of slugs stained with Nitro Blue Tetrazolium and succinate after centrifugation through a Urografin gradient. This photograph was prepared from colour-sensitive film. The slug stage of *D. discoideum* viewed from (c) above and (d) the side is also shown.

EXPLANATION OF PLATE 2

Electron micrographs of sections of cells from the slug stage of *D. discoideum*. (a) Light cells from a Urografin gradient. (b) Heavy cells from a Urografin gradient. (c) Transverse section of the anterior portion of an intact slug. The arrows indicate structures found only in the heavy cells and the anterior portion of the slug.

Table 1. *Enzymic composition of cell fractions*

Cell fraction I extract contained 0.56 mg. of protein/ml.; cell fraction II extract contained 0.89 mg. of protein/ml.

Enzyme	Specific activity in extracts		
	From cell fraction I (heavy)	From cell fraction II (light)	I/II ratio
Alkaline phosphatase	39.6	26.8	1.5
N-Acetylglucosaminidase	20.5	214	0.10
UDP-glucose pyrophosphorylase	8.6	15.1	0.57
Malate dehydrogenase	442	1125	0.39
Citrate synthase	4.9	114	0.04

purple, suggesting that the heavy cells are derived from the anterior portion of the slug.

When serial transverse sections of fixed and uranyl acetate-osmic acid-stained slugs were examined in the electron microscope, cells in the anterior portion of the slug were seen to contain an unusual and characteristic structure. This structure, or structures, consists of sheets of double membrane on which are arranged, in a highly regular fashion, spherical bodies with the dimensions of ribosomes. The membranes appear to connect vesicular structures with the outer membranes (Plate 2c, arrows). When heavy and light cell fractions are similarly fixed and stained and sections thereof examined in the electron microscope all the cells in the heavy fraction contain these structures (Plate 2b, arrows), whereas none of the cells in the light fraction contains these structures (Plate 2a). This observation again suggests that the heavy cells originate from the anterior portion of the slug and the light cells from the posterior portion.

DISCUSSION

Density gradients of Urografin have been used by others to separate subcellular components (Schatz, Haslbrummer & Tuppy, 1964) and bacterial cells (Tamir & Gilvarg, 1966). We have extended this technique to separate two distinct cell populations from a tissue-like structure, the slug stage of *D. discoideum*.

In the classical papers describing this organism Raper (1940) showed that the slug has a definite polarity. Using grafting techniques he was further able to show that the anterior cells of the slug become the stalk cells of the fruiting body and the posterior cells the spores of the fruiting body. It is thus possible to divide the slug into two distinct regions, an anterior region (about one-third of the whole) of pre-stalk cells and a posterior region of pre-spore cells. Histochemical techniques have shown that several compounds such as polysaccharides (Bonner, Chiquoine & Kolderie, 1955),

antigenic determinants (Takeuchi, 1963; Gregg, 1965) and respiratory activities (Miné & Takeuchi, 1967) are unevenly distributed along the longitudinal axis of the slug. We have extended these cytological observations to the level of fine structure and have shown that the anterior third of the slug is uniquely characterized by the possession of structures of the type shown in Plate 2(c). It is not, perhaps, unreasonable to suppose that the presence of such structures would affect the density of the cell containing them, and we attempted to use isopycnic gradient techniques to separate the anterior cells from the posterior cells. Gradients prepared from sucrose and Ficoll proved useless for this purpose, since these materials dehydrated the cells, which consequently formed pellets at all concentrations. Faust & Filosa (1959) have reported that *D. discoideum* myxamoebae are impermeable to sucrose. Urografin provides, however, a suitable compound for making density gradients, giving stable gradients in the appropriate density range and not affecting the viability or biochemical capacities of the cells. Preliminary experiments have also shown that mixtures of hydrophobic liquids such as phthalate esters can also give density gradients suitable for the isopycnic separation of eucaryotic cells. As shown in Plate 1(a) Urografin gradients have proved suitable for isolating from the slug two distinct cell populations, which differ in a number of enzymic activities, histochemical behaviour and fine structure. We believe that these two populations represent the anterior and posterior cells of the slug and thus (Raper, 1940) those cells that would have become respectively the stalk and spore cells of the fruiting body. The absence from the slug of any cells of intermediate density, cytological appearance or histochemical behaviour poses, in an acute form, the problem of how an initially homogeneous population of cells (the myxamoebae) can give rise to a structure, such as the slug, containing two distinct and very different cell types arranged sequentially along a longitudinal axis. This process is especially interesting

since it is readily reversible by the provision of foodstuffs (bacteria), is dependent on the maintenance of the integrity of the slug (since both light and heavy cells will give rise to normal, albeit small, fruiting bodies) and is accomplished in the absence of any DNA synthesis or change in cell number. Clearly in the slug there must exist some form of cell-cell interaction as a result of which the two different cell populations are maintained in correct numerical and spatial relationship to one another. The nature of this interaction and the manner in which the cell heterogeneity in the slug is first established and then maintained remain unexplained. The establishment of the cell heterogeneity in the slug, which is clearly associated with the aggregation process, does, however, show a curious similarity to hormonal control mechanisms in higher organisms, since it appears to be brought about by concentration gradients of cyclic-AMP (Konijn, Barkley, Chang & Bonner, 1968).

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